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Oligosaccharide conjugate vaccines

Abstract

The present invention relates to an improved method for producing oligosaccharide conjugate vaccines. In an additional aspect of the invention, oligosaccharide vaccines are produced which elicit a monospecific and homogeneous immune response to capsular polysaccharide. A specific embodiment of the invention provides for vaccines which induce immunity to prevalent serotypes of Streptococcus pneumoniae.

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Claims

What is claimed is:

1. A method for producing a covalent conjugate of an oligosaccharide and a carrier protein, comprising the following steps:

(i) reacting an oligosaccharide having a terminal reducing group with diaminomethane in the presence of pyridine borane such that reductive animation occurs; and

(ii) reacting the animated oligosaccharide product of (i) with a molecular comprising two functional groups, one of which is capable of reacting with the terminal group of the activated oligosaccharide and the other which is capable of reacting with said carrier protein; and

(iii) reacting the activated oligosaccharide product of (ii) with said carrier protein such that conjugation occurs.

2. The method according to claim 1 in which reductive animation is performed at a temperature of about 100.degree. C.

3. The method according to claim 2 in which the reductive animation reaction is allowed to proceed for about 15 minutes.

4. The method according to claim 1 in which the reaction with pyridine borane is performed at a temperature of about 50.degree. C.

5. The method according to claim 4 in which the reaction with pyridine borane is allowed to proceed for about 48 hours.

6. The method according to claim 1 in which the molecule comprising two functional groups of step (ii) is a diester.

7. The method according to claim 1 in which the molecule comprising two functional groups of step (ii) is a diester of adipic acid or a diester of succinic acid.

8. The method according to claim 7 in which the molecule comprising two functonal groups of step (ii) is the succinimidyl diester of succinic acid.

9. The method according to claim 7 in which the molecule comprising two functional groups of step (ii) is the succinimidyl diester of adipic acid.

10. The method according to claim 1, 2, 3, 4, 5, 6, 7, 8, or 9 in which the reaction of step (ii) is performed at about 4.degree. C. for about 2 hours.

11. A covalent conjugate between oligosaccharides and a carrier protein produced by a method comprising the steps of:

(i) hydrolyzing a polysaccharide to produce oligosaccharides which have at least one terminal reducing group; and

(ii) reacting said oligosaccharides with diaminomethane in the presence of pyridine borane such that reduce animation occurs; and

(iii) reacting the animated oligosaccharide product of (i) with a molecule comprising two functional groups, one of which is capable of reacting with the terminal group of the activated oligosaccharide and the other which is capable of reacting with said carrier protein; and

(iv) reacting the activated oligosaccharide product of (ii) with said carrier protein such that conjugation occurs.

12. The covalent conjugate of claim 11 in which the molecule comprising two functional groups of step (iii) is a diester.

13. The covalent conjugate of claim 12 in which the molecule comprising two functional groups of step (iii) is a diester of adipic acid.

14. The covalent conjugate of claim 12 in which the molecule comprising two functional groups of step (iii) is the succinimidyl diester of succinic acid.

15. The covalent conjugate of claim 12 in which the molecule comprising two functional groups of step (iii) is the succinimidyl diester of adipic acid.

16. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9 in which the oligosaccharide is derived from Streptococcus pneumoniae capsular polysaccharide.

17. The method of claim 16 in which the oligosaccharide is derived from Streptococcus pneumoniae having a selected serotype selected from the group consisting of types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F.

18. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9 in which the oligosaccharide is derived from capsular polysaccharide from a bacterium selected from the group consisting of Haemophilus influenzae, Neisseria meningitidis, Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli, Streptococcus mutans, Cryptococcus neoformans, Klebsiella pneumoniae, and Staphylococcus aureus.

19. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9 in which the carrier protein is CRM.sub.197.

20. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9 in which the carrier protein is selected from the group consisting of Salmonella flagellin, Haemophilus pillin, Haemophilus 15 kDa, 28-30 kDa, or 40 kDa membrane protein, Escherichia coli heat labile enterotoxin LTB, diphtheria toxin, tetanus toxin, cholera toxin, rotavirus VP7 protein, and respiratory syncytial virus F or G protein.

21. The covalent conjugate between oligosaccharide and carrier protein of claim 11, 12, 13, 14 or 15 in which the oligosaccharide is derived from Streptococcus pneumoniae capsular polysaccharide.

22. The covalent conjugate of claim 21 in which the oligosaccharide is derived from Streptococcus pneumoniae having a serotype selected from the group consisting of types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F.

23. The covalent conjugate between oligosaccharide and carrier protein of claim 11, 12, 13, 14, or 15 in which the oligosaccharide is derived from capsular polysaccharide from a bacterium selected from the group consisting of Haemophilus influenzae, Neisseria meningitidis, Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli, Streptococcus mutans, Cryptococcus neoformans, Klebsiella pneumoniae, and Staphylococcus aureus.

24. The covalent conjugate between oligosaccharide and carrier protein of claim 11, 12, 13, 14, or 15 in which the carrier protein is CRM.sub.197.

25. The covalent conjugate between oligosaccharide and carrier protein of claim 11, 12, 13, 14, or 15 in which the carrier protein is selected from the group consisting of Salmonella flagellin, Haemophilus pillin, Haemophilus 15 kDa, 28-30 kDa, or 40 kDa membrane protein, Escherichia coli heat labile enterotoxin LTB, diphtheria toxin, tetanus toxin, cholera toxin, rotavirus VP7 protein, and respiratory syncytial virus F or G protein.

26. The covalent conjugate of claim 21 in which the oligosaccharide is derived from Streptococcus pneumoniae having a serotype selected from the group consisting of types 6A, 14, 19F and 23F.

27. The covalent conjugate between oligosaccharide and carrier proteins of claim 26 in which the carrier protein is CRM.sub.197.

Description

1. INTRODUCTION

The present invention relates to an improved method for producing oligosaccharide conjugate vaccines. In an additional aspect of the invention, oligosaccharide vaccines are produced which elicit a monospecific and homogeneous immune response to capsular polysaccharide. A specific embodiment of the invention provides for vaccines which induce immunity to prevalent serotypes of Streptococcus pneumoniae which may be particularly important for use in pediatric patients as well as the elderly and those with reduced immunity due to infirmity or disease (including for example, AIDS patients).

2 BACKGROUND OF THE INVENTION

2.1. DISEASES CAUSED BY STREPTOCOCCUS PNEUMONIAE

The pneumoccus (Streptococcus pneumoniae) is a gram-positive encapsulated coccus that usually grows in pairs or short chains. In the diplococcal form, the adjacent margins are rounded and the opposite ends slightly 0 organisms a lancet shape. pointed, giving the

Pneumococci may be divided into serotypes based on the complex polysaccharides which form their capsules. 84 serotypes have been identified by exposure to type-specific antiserum, the Neufeld quelling reaction. All are pathogenic for human beings, but types 1, 3, 4, 7, 8, and 12 are encountered most frequently in clinical practice. Types 6, 14, 19, and 23 often cause pneumonia and otitis media in children but are less common in adults (Austrian, 1983, in "Harrison's Principles of Internal Medicine", Petersdorf et al., eds., 10th Edition, McGraw Hill Book Co., New York pp. 918-922). Notably, the pneumococcus is one of the three primary pathogens responsible for pneumonia, sepsis, and meningitis in children (McMillan, 1982, in "Core Textbook of Pediatrics, Kaye et al., eds., Second Edition, J. B. Lippincott Co., Philadelphia, p. 498).

2.2. Pneumococcal Vaccines

Individuals at higher than average risk of developing pneumococcal infections include patients with chronic systemic illnesses such as heart disease, chronic bronchopulmonary disease, hepatic disease, renal insufficiency, and malignancy. It is recommended that these individuals be vaccinated against pneumococcal infection . this purpose, twenty-three vaccines comprising the capsular polysaccharides of pneumococcal types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, IIA, 12F, 14, 15B, 17F, 18C, 19A, 9F, 20, 22F, 23F, and 33F (which include serotypes or groups responsible for 90 percent of serious pneumoccal disease in the United States and the rest of the world) are available (Pneumovax.RTM. Merck, Sharpe & Dohme, and PnuImmune.RTM., Lederle Laboratories). The efficacy of this vaccine in children is questionable, as, in children younger than 6 years, immunologic responsiveness to various capsular antigens develops at different times as a result of maturational characteristics of the immune system, and protection may be of shorter duration than that observed in adults (Harrison et al., ibid). Although relatively few neumococcus serotypes are believed to account for the majority of pediatric pneumococcal infections (Gray et al., 1979, J. Infect. Disease 140:979-983), these include types for which the maturation of the human antibody response to purified capsular polysaccharides used as vaccines is slowest (Anderson and Betts, 1989,

Pediatric Infec. Dis. J. 8:S50-S53; Borgono et al., 1978, Proc. Soc. Exp. Biol. Med. 157:148-154).

2.3. Conjugate Vaccines

Immune responsiveness in human infants to Haemophilus influenzae b capsular polysaccharide has been achieved by coupling the capsular antigen to carrier proteins to produce a "conjugate" vaccine; it is believed hat T lymphocyte helper effects are induced by the carrier protein and are responsible for the development of immunity (Robbins et al., 1984, in "Bacterial Vaccines," Germanier, ed. Academic Press, New York, pp. 289-316) See also: Cruse & Lewis, 1989 in "Conjugate Vaccines" eds. Cruse & Lewis, Basel, pp. 1-10.. A similar approach has been directed toward producing pneumococcal vaccines.

2.3.1. Intact Capsular Polymers as Antigens in Vaccines

Various investigators have isolated and purified intact capsular polymers which may be useful in or as vaccines. For example, U.S. Pat. No. 4,220,717 describes a process for the isolation and purification of immunologically active polyribosyl ribitol phosphate (PRP) from the capsular polymer of H. influenzae b. Additionally, U.S. Pat. No 4,210,641 relates to polysaccharide extracts of H. influenzae having an apparent molecular weight greater than 200,000 daltons and composed principally of galactose, glucose and mannose and containing a small amount of osamines.

Several researchers have utilized these and other intact capsular polymers in formulations to achieve better immunological responses. For example, U.S. Pat. No. 4,196,192 discloses a vaccine containing purified intact and a whole cell Bordetella pertussis vaccine formulation. This approach to increasing immunogenicity resulted in enhanced levels of anti-PRP and anti-pertussis antibodies in young mammals.

2.3.2 Use of Carrier Proteins to Make Antiserum to Haptens

Carrier proteins can do more than enhance the immunogenicity of conjugated capsular polymers; they can also render haptens immunogenic. Haptens are defined as molecules that can bind specifically to an antibody or lymphocyte receptor but may not themselves induce an immune response (i.e. they are not immunogenic). To evoke an immune response, small/low molecular weight or poorly immunogenic molecules, termed haptens, must generally first be coupled to a larger molecule, or carrier, which is usually a heterologous protein. Injection of the hapten carrier complex into an animal will then give rise to the production by B lymphocytes of antibodies, some of which will be capable of specifically binding to the free, uncoupled hapten molecule.

Among the earliest haptens to be studied were azo dye compounds such as aniline and o-aminobenzoic acid. Landsteiner and Lampl (1918, Z. Immun. Forsch 26:293) coupled these compounds by diazotization to serum proteins. When injected with these artificially prepared azo-proteins, rabbits developed precipitating antibodies that were specific for the attached chemical moieties.

Other examples of haptenic compounds are dinitrophenol, which becomes immunogenic upon coupling as the dinitrophenyl (DNP) group to bovine serum albumin or to bovine gamma globulin (BGG), and lysergic acid diethylamide. Even formaldehyde has been shown to behave as a hapten; persons exposed to formaldehyde vapors from products or in laboratories have become "sensitized" to the compound, following the formulation of their endogenous macromolecules in vivo.

Haptenic behavior is not limited to small organic molecules, and polypeptide hormones up to the size of insulin are often poorly, if at all, immunogenic. To obtain high antibody titers to these hormones it is thus necessary to conjugate them to a carrier molecule (or to create larger molecules by crosslinking many of

these polypeptides together).

The involvement of the carrier molecule is especially interesting in that the carrier plays more than a mere transport role. Ovary and Benaceraff (1963, Proc. Soc. Exp. Biol. Med. 114:723) showed this by injecting rabbits with DNP-BCG. Injection of many immunogenic materials into animals will produce an immunological "memory" of the exposure. When a second injection is given later, there is thus a much more vigorous immune response. Indeed, when Ovary and Benaceraff injected DNP-BCG again, there was a strong, secondary response that led to markedly elevated levels of antibodies directed against both DNP and BCG. But when the second injection was instead made with DNP-egg albumin, a much weaker anti-DNP antibody response was noted. The difference in response was due to what has been called the carrier effect, and it appears to involve helper T lymphocytes.

Preliminary evidence indicates that all proteins may not be equally effective carrier proteins for a given hapten. Robbins, et al. (Infect. Immun. 40:245-256) have presented data on experimental protein-polysaccharide conjugate vaccines in which the same polysaccharide hapten was conjugated to different protein carriers and the antibody response to the hapten was quantified. Significant differences were noted in the amount of anti-hapten antibody generated, indicating a major role for the carrier.

With respect to pneumococcal vaccines in particular Lin, Lee (1982, Immunology 46:333) studied immune responses in adult and young mice exposed to type 6A and 19F polysaccharides as well as 19F conjugated to protein. Significantly higher IgM and IgG2 antibody titers were induced in mice receiving 19F polysaccharide-protein conjugates than in the control group receiving 19F polysaccharide alone.

2.3.3. Vaccines Containing Conjugates

Other researchers have studied conjugation of capsular polymers to carrier proteins in an effort to enhance antibody formation by the so-called "carrier effect". For example, Schneerson et al., Journal of Experimental Medicine 152:361-376 (1980) describes H. influenzae b polymer-protein conjugates disclosed to confer immunity to invasive diseases caused by H. influenzae b. The reference documents the age-related immunological behavior of capsular polymers in infants and seeks to overcome this age-dependence by conjugation of the intact capsular polymer with a variety of proteins, including serum albumins, Limulus polyphemus hemocyanin and diphtheria toxin. The method of conjugation involves the use of a linking agent such as adipic dihydrazide.

Geyer et al., Med. Microbiol. Immunol. 165:171-288 (1979), prepared conjugates of certain Klebsiella pneumoniae capsular polysaccharide fragments to a nitrophenyl-ethylamine linker by reductive amination, and the derivatized sugar was then attached to proteins using azo coupling.

U.S. Pat. No. 4,057,685 by McIntire, filed May 9, 1974 relates to an Escherichia coli lipopolysaccharid reduced toxicity covalently coupled to a protein antigen by reaction with haloacyl halide.

U.S. Pat. No. 4,356,170 by Jennings et al., filed May 27, 1981, issued Oct. 26, 1982, relates to the production of polysaccharide-protein conjugates by reductive amination.

Anderson (1983, Infection and Immunity 39:233-238) relates to conjugates between oligosaccharides from Haemophilus influenzae type b capsular polysaccharide and CRM.sub.197, a nontoxic but antigenically identical variant diphtheria toxin.

Snippe et al. 1983, Infection and Immunity 42:842-844), relates to a semisynthetic vaccine to Streptococcus pneumoniae type 3 in which a hexasaccharide isolated from a partial acid hydrolysate of the

capsular polysaccharide S3 was coupled to stearyl amine by reductive amination and then incorporated into liposomes. The resulting conjugate/liposome vaccine was observed to induce protection to S. pneumoniae type 3 in mice.

U.S. Pat. No. 4,663,160 by Tsay et al., filed Mar. 14, 1983, issued May 5, 1987, relates to bacteria in which a detoxified polysaccharide from a gram-negative bacterium is covalently coupled to a detoxified protein from the same species of gram-negative bacterium, by means of a 4-12 carbon moiety.

U.S. Pat. No. 4,619,828 by Gordon, filed Jan. 5, 1984, issued Oct. 28, 1986, relates to conjugates between polysaccharide molecules from pathogenic bacteria such as Haemophilus influenzae b, Streptococcus pneumoniae, Neisseria meningitidis, and Escherichia coli and T cell dependent antigens such as diphtheria and tetanus toxoids.

U.S. Pat. No. 4,808,700 by Anderson and Clements, filed Aug. 10, 1984, issued Feb. 28, 1989, and U.S. Pat. No. 4,761,283 by Anderson, filed Mar. 28, 1986,--issued Aug. 2, 1988, relate to the covalent attachment of capsular polymer fragments to bacterial toxins, toxoids, or binding subunits by means of reductive amination.

U.S. Pat. No. 4,711,779 by Porro et al., filed Jul. 2, 1986, issued Dec. 8, 1987, relates to glycoprotein conjugate vaccines having trivalent immunogenic activity and comprising antigenic determinants from the capsular polysaccharides of a gram positive bacterium and a gram negative bacterium, as well as either CRM.sub.197, tetanus toxoid, or pertusis toxin.

2.3.4. Method for Preparing Conjugate Vaccines

The preparation of conjugate vaccines, in which capsular polysaccharide haptens are linked to carrier proteins, entails the following procedures:

(i) capsular polysaccharide must be prepared

(ii) if a fragment of the polysaccharide is to be used, it must be separated from intact polysaccharide

(iii) saccharide must be activated, or rendered amenable to conjugation, i.e. moieties capable of covalently bonding to protein must be generated

(iv) saccharide is conjugated to protein. Various methods known in the art for accomplishing these four steps are listed in Table I.

TABLE I

Reference	Preparation of Polysaccharide	Cleavage of Polysaccharide	Activation of Polysaccharide	Conjugation to Protein
U.S. Pat. No. 4,356,170 by Jennings, filed May		Employed Periodic Acid to generate aldehyde groups		Reductive Amination using cyanoborohydride

27, 1981 issued October 25, 1982 U.S. Pat. No. 4,663,160 by Tsay et al., filed March 14, 1983, issued May 5, 1987.	Employed Peroidic Acid to generate aldehyde groups	1) 4-12 carbon moiety linked to protein in the presence of a condensing agent, e.g. carbodiimide ii) Polysaccharide linked to protein derivatized with 4-12 carbon moiety via a Schiff's' base reaction in the presence of a reducing agent, e.g. cyanoborohydride
U.S. Pat. No. 4,619,828 by Gordon, filed January 5, 1984, issued October 28, 1986	Polysaccharides adjusted by heat treatment to a molecular size between 200,000 and 2,000,000 daltons	*Cyanogen bromide *Conjugated via a spacer bridge of 4-8 carbon atoms, as would exist in the adipic acid hydrazide derivative of the protein
U.S. Pat. No. 4,808,700 by Anderson and Clements, filed August 10, 1984, issued February 28, 1989	A variety of methods are used to produce antigenic fragments with reducing end, e.g. limited oxidative cleavage by periodate, hydrolysis by glycosidases, or acid hydrolysis	Conjugation via at least one reductive amination in the presence of cyanoborohydride (approximately 2-3 weeks)
Section 6.5 of the above, entitled "Conjugation of Capsular Polymer Fragment of Streptococcus Pneumoniae to CRM.sub.197 "	Danish type 6A, Eli Lilly Co. Acid hydrolysis in 0.1N HCl for 10 minutes at 100.degree. C. to generate reducing CRM.sub.197 in phosphate buffer using sodium cyanoborohydride for 18 days at 37.degree. C.	Conjugated to
U.S. Pat. No. 4,711,779 by Porro and Constantino, filed July 2, 1986,	Acid hydrolysis at 100.degree. for 6-40 hours. Haptens suitable have a molecular weight of	Activated by introducing primary amino groups into the terminal reducing	Conjugated to toxoid in the presence of

			organic solvent,
issued December 8,	1000 to 2000 daltons	groups (e.g. using	e.g. dimethyl-
1987.		sodium cyanoboro-	sulfoxide
		hydride) with subsequent	conversion to esters
		(e.g. in the	presence of adipic
		acid derivatives)	
for streptococcus	Acid hydrolysis at	Activated in	Conjugated to
pneumoniae Type	100.degree. C. for 39 hours	ammoniacal buffer	CRM.sub.197 in the
6A		in the presence of	presence of di-
		sodium cyanoborohydride	methylsulfoxide at
		(to introduce primary	room temperature
		amino groups) for two	for 15 hours
		weeks; converted to	
		corresponding mono-	
		functional esters in an	
		aqueous solution of	
		dimethylsulfoxide con-	
		taining disuccinimidyl	
		ester of adipic acid	
		for four hours	

3. SUMMARY OF THE INVENTION

The present invention relates to the covalent attachment of oligosaccharides derived from bacterial capsular polysaccharides to carrier proteins using a novel process.

This process permits the efficient synthesis of glycoconjugates at production rates significantly faster than currently employed methods. The glycoconjugates of the invention may be used in vaccine formulations, and have been shown to be immunogenic.

In a particular embodiment, the present invention relates to production of glycoconjugates which incorporate oligosaccharides derived from Streptococcus pneumoniae capsular polysaccharides. The method of the invention results in the efficient production of high yields of S. pneumoniae glycoconjugates which may be used in vaccine formulations of particular relevance to the pediatric population, in which a large proportion of major illnesses are associated with S. pneumoniae infection. Immunogenic conjugates have been found to be less age dependent than

alone, and are useful for the active capsular polymers immunization of very young warm-blooded mammals against systemic infections by the respective encapsulated bacteria.

In a further aspect of the invention, the glycoconjugates of the invention have, surprisingly, been found to elicit a monospecific and homogenes immune response, which may advantageously avoid the generation of autoimmune reactions and related post-vaccination syndromes.

Importantly, the immunogenic conjugates of the invention do not contain potentially toxic linking agents, such as adipic dihydrazide or p-nitro-phenylethylamine, which have been used in conjugating carbohydrate to protein.

3.1. Abbreviations and Definitions

CRM.sub.197	a non-toxic protein antigenically cross-reactive with diphtheria toxin
DMSO	dimethylsulfoxide
DP	degree of polymerization
MIC	minimum inhibitory concentration
SD	substitution degree
SIDEA	succinimidyldiester of adipic acid
SIDES	succinimidyldiester of succinic acid

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. General strategy for synthesis of oligosaccharide-protein conjugates.

A. High molecular weight polysaccharides are acid hydrolyzed to yield oligosaccharides of an average molecular weight of 2.5.times.10.sup.3.

B. Oligosaccharides are (1) activated by reaction with diaminoethane [H.sub.2 N(CH.sub.2).sub.2 NH.sub.2]at pH=9, reduced with pyridine borohydride (PyBH.sub.3), then (2) reacted with the succinimidyl diester and adipic acid (SIDEA) in dimethylsulfoxide (DMSO).

C. Activated oligosaccharides are coupled to carrier protein via lysine residues.

FIG. 2. Use of a "tailored" spacer in the coupling procedure.

A. Glycoconjugate formed by earlier procedure as described by Porro et al., (1985), Mol. Immunol. 22:907-919, with amide linkage (arrow) between oligosaccharide and adipic acid four carbon linker. Total length of spacer is approximately 10.4 .ANG..

B. Glycoconjugate formed according to the present invention in which a two carbon residue (arrow, formed by diaminoethane), and an amide linkage, exists between oligosaccharide and succinic acid two carbon linkers formed by reaction with SIDES. Total length of spacer is approximately 10 .ANG..

C. Glycoconjugate formed according to the present invention in which a two carbon residue (arrow, formed by diaminoethane), and an amide linkage, exists between oligosaccharide and adipic acid four carbon residue formed by reaction with .SIDEA. Total length of spacer is approximately 14.5 .ANG..

FIG. 3 Efficiency of conjugation of CRM.sub.197 to activated oligosaccharides containing adipic acid versus succinic acid derivative spacers. SDS-polyacrylamide gel electrophoresis of products of conjugation reactions (silver stained).

A. Lane 1: Molecular Weight Standards (92.5 K, 66.2 K, 45.0 K, 31.0 K, 21.5 K).

Lane 2: CRM.sub.197 (1 .mu.g) reference.

Lane 3: Conjugated oligosaccharide 6A-CRM.sub.197 with succinic acid as spacer (2 .mu.g) (ratio 1:1 monoester/total amino groups of CRM197 in 50% DMSO).

Lane 4: Conjugated oligosaccharide 6A-CRM.sub.197 with succinic acid as spacer (2 .mu.g) (ratio: 1:2 monoester/total amino groups of CRM.sub.197 in 50% DMSO).

Lane 5: Conjugated oligosaccharide 6A-CRM.sub.197 with adipic acid as spacer (2 .mu.g) (ratio: 1:2 monoester/total amino groups of C.sub.197 in 50% DMSO).

Lane 6: Conjugated oligosaccharide 14-CRM.sub.197 with succinic acid as spacer (2 .mu.g) (ratio: 1:4 monoester/total amino groups of C in 50% DMSO).

Lane 7: Conjugated oligosaccharide 19F-CRM.sub.197 with succinic acid as spacer (2 .mu.g) (ratio: 1:4 monoester/total amino groups of C in absence of 50% DMSO).

Lane 8: Conjugated oligosaccharide 23F-CRM.sub.197 with succinic acid as spacer (2 .mu.g) (ratio: 1:2 monoester/total amino groups of CRM.sub.197 in 50% DMSO).

Lane 9: CRM.sub.197 (1 .mu.g) reference.

B. Lane 1: CRM.sub.197 (1 .mu.g) reference.

Lane 2: CRM.sub.197 reference (1 .mu.g, different lot compared to Lane 1)

Lane 3: Conjugated oligosaccharide 23F-CRM.sub.197 with adipic acid as spacer (2 .mu.g) (ratio: 1:2 monoester/total amino groups of CRM.sub.197 in 50% DMSO).

Lane 4: Molecular Weight Standards (92.5 K, 66.2 K, 45.0 K, 31.0 K., 21.5 K).

Lane 5: Conjugated oligosaccharide 23F-CRM.sub.197 with adipic acid as spacer (2 .mu.g) (ratio: 1:2 monoester/total amino groups of CRM:M.sub.197 in 50% DMSO).

Lane 6. CRM.sub.197 (1 .mu.g) reference CRM.sub.197 reference (1 .mu.g, different lot compared to Lane 1)

Lane 7: Conjugated oligosaccharide 6A-CRM.sub.197 with adipic acid as spacer (2 .mu.g)

C. Lane 1: Molecular Weight Standards (92.5 K, 66.2 K, 45.0 K, 31.0 K, 21.5 K)

Lane 2: CRM.sub.197 (1 .mu.g) reference.

Lane 3: Conjugated oligosaccharide 6A-CRM.sub.197 with adipic acid as spacer (2 .mu.g)

Lane 4: Conjugated oligosaccharide 14-CRM.sub.197 with adipic acid as spacer (2 .mu.g)

Lane 5: Conjugated oligosaccharide 19F-CRM.sub.197 with adipic acid as spacer (2 .mu.g)

Lane 6: Conjugated oligosaccharide 23F-CRM.sub.197 with adipic acid as spacer (2 .mu.g)

Lane 7: Molecular Weight Markers (92.5 K, 66.2 K, 45.0 K, 31.0 K, 21.5 K)

FIG. 4. Rabbit IgG response to S. pneumoniae oligosaccharide 6A-CRM.sub.197 conjugates. Inhibition-ELISA analysis of affinity value of IgG isotype induced to the capsular polysaccharides.

A. Type 6A capsular polysaccharide

B. Type 6A oligosaccharide (DP =10)in free form or conjugated to CRM.sub.197

C. Type 14 oligosaccharide DP=12) activated by molecular spacer or conjugated to C

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the covalent attachment of oligosaccharides derived from bacterial capsular polysaccharides to carrier proteins; the method of the invention generates novel glycoconjugates via a novel process.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following sections:

(i) Preparation of Oligosaccharides

(ii) Activation of Oligosaccharides

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Preparation of Oligosaccharides

High molecular weight capsular polysaccharide may be purchased commercially (American Type Culture Collection (Rockville, MD)) or obtained by the methods described by Porro et al., 1983, J. Biol. Stand. 11:65-71. Any polysaccharide may be used, including, but not limited to, those found in the capsules of Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Escherichia coli. Salmonella typhi, Streptococcus mutans, Cryptococcus neoformans, Klebsiella pneumoniae, Staphylococcus aureus, and Pseudomonas aeruginosa.

Antigenic fragments with at least one reducing end can be generated from capsular polymers by a variety of methods, depending upon the structural features of the particular capsular polymer. Limited oxidative cleavage by periodate (or related reagents) will leave aldehydic termini; such an approach will be limited to polymers having vicinal dihydroxy groups on a non-cyclic residue. Hydrolysis of a glycosidic linkage produces a reducing sugar terminus. Such hydrolysis can be most specifically accomplished enzymatically by glycosidases, but this application would be restricted to a relatively few capsular polymers, e.g. Streptococcus pneumoniae 8, for which glycosidases are known. Acidic hydrolysis is commonly used for

hydrolysis of glycosidic linkages. The utility of this approach would be limited if the polymer contains acid-sensitive non-glycosidic linkages or if the polymer contains acid-sensitive branch linkages important to the antigenic specificity.

In specific embodiments of the invention, S. pneumoniae type 6A capsular polysaccharide may in approximately 10.sup.-2 M acetic acid at about 100.degree. C. for about 30 hours; S. pneumoniae type 14 capsular polysaccharide may be hydrolyzed in approximately 0.5 M trifluoroacetic acid at about 70.degree. C. for about 7 hours; S. pneumoniae type 19F polysaccharide may be hydrolyzed in approximately 10.sup.-2 M acetic acid at about 50.degree. for about 48 hours; and S. pneumoniae type 23F polysaccharide may be hydrolyzed in approximately 0.25 M trifluoroacetic acid at about 70.degree. C. for about 3 hours.

According to the invention, oligosaccharides to be conjugated to protein preferably consist of between three and six repeating units (or between about ten and thirty monosaccharide residues), and more preferably consist of between three and four repeating units (or about fifteen monosaccharide residues) as oligosaccharides of this length, incorporated into glycoconjugates, have been shown to be optimally immunogenic.

5.2. Activation of Oligosaccharides

Oligosaccharides may be activated by a process of reductive amination followed by reaction with a bifunctional molecule, such as, but not limited to, a diester. An outline of the method of the invention is presented in FIG. 1 and Table II, which compares the method of the present invention with the method described in Porro et al., 1985, Mol. Immunol. 22:907-919. Note that the time of activation using the former procedure was 7-14 days; this has been shortened, according to the present invention, to 15 minutes. Note also that the time of reduction using the former procedure was 7-14 days; this has been shortened, according to the present invention, to 48 hours. Accordingly, the present invention requires 12-26 fewer days to complete than the former process. This is an important advantage, as exposing carbohydrates to elevated temperatures, such as 50.degree. C., may lead to degradation.

TABLE II

Chemical Activation of the end-reducing unit of S. pneumoniae Oligosaccharides		
Parameters	Former Procedure	Present Procedure
Introduced Group	NH.sub.2	NH(CH.sub.2).sub.2 NH.sub.2
Reagent (pH)	Ammoniacal buffer (9)	Diaminoethane (9)
Temperature of activation	50.degree. C.	100.degree. C.
Time of activation	7-14 days	15 minutes
Reducing agent	Na cyanoborohydride	Pyridine borane
Temperature of reduction	50.degree. C.	50.degree. C.
Time of reduction	7-14 days	48 hours
Resulting product	Oligo-NH.sub.2	Oligo-NH(CH.sub.2).sub.2 NH.sub.2
Activating Bi-		

	SIDEA (Succinimidyl	
		SIDES or SIDEA
functional	Spacer	
	diester of adipic	
		(succinimidyl diester
	acid	of succinic or adipic
		acid)
Temperature	of	
	25.degree. C.	4.degree. C.
reaction		
Time of reaction		
	4 hours	2 hours
Resulting	Oligo-NH-Monoester	
		Oligo-NH(CH.sub.2).sub.2 NH-
product		monoester
Efficiency	of	
	25-30%	70%
Reaction		

According to the method of the invention, reductive amination of the end-reducing unit of an oligosaccharide is performed using a molecule containing two amino groups. In a preferred embodiment of the invention, reductive amination is accomplished by reacting a given molar amount of oligosaccharide with a diaminoethane solution in 10X molar excess in 0.2M KH.sub.2 PO.sub.4 at about pH=9 at a temperature of approximately 25.degree.-100.degree. C., and preferably 100.degree. C. for between about 1-60 minutes, and preferably about 15 minutes. After that a molar amount of pyridine borane equivalent to 25 times the molar concentration of oligosaccharide in the preparation may be added and reaction is performed at between about 25.degree.-100.degree. C., and preferably about 50.degree. C. for between about 1 and 72 hours about 48 hours.

The resulting product of the reductive amination reaction may then be reacted with a bifunctional molecule, each functional group being capable of reaction with either the terminal amino group of the activated oligosaccharide and amino groups present in the structure of the carrier protein, such that the bifunctional molecule may serve to link together the oligosaccharide and the carrier protein. In a preferred embodiment of the invention, the bifunctional group is a diester, and is, more particularly, a diester of adipic acid, which has been shown to be associated with more efficient glycosylation of protein. In a preferred, specific embodiment of the invention an oligosaccharide, having been subjected to reductive amination as described supra, is further reacted with a succinimidyl diester of succinic or, more preferably, adipic acid; this reaction may best be performed with the aminated oligosaccharide at a molar concentration (as amino groups) equivalent to about one-fifth of the molar concentration of SIDEA (or SIDES) in a solution of dimethylsulfoxide (DMSO) at between about 0.degree. and 25.degree. C., and preferably about 4.degree. C. for between about 0.5 and 5 hours and preferably about 2 hours. The activated oligosaccharide may then be collected by precipitation using 1,4 dioxane (80% v/v), which also leaves in the supernatant the excess of SIDEA (or SIDES).

5.3. Conjugation of Oligosaccharides to Protein

Proteins which may be utilized according to the invention include any protein which is safe for administration to young mammals and which may serve as an immunologically effective carrier protein. In particular embodiments, cell surface proteins, membrane proteins, toxins and toxoids may be used. Criteria for safety would include the absence of primary toxicity and minimal risk of allergic reaction. Diphtheria and tetanus toxoids fulfill these criteria; that is, suitably prepared, they are non-toxic and the incidence of allergic reactions is acceptably low. Although the risk of allergic reaction may be significant for adults, it is

minimal for infants. According to additional particular embodiments of the invention, appropriate carrier proteins include, but are not limited to Salmonella flagellin, Hemophilus pilin, Hemophilus 15 kDa, 28-30 kDa, and 40 kDa membrane proteins, Escherichia coli heat labile enterotoxin LTB, cholera toxin, and viral proteins including rotavirus VP7 and respiratory syncytial virus F and G proteins.

In the "carrier effect" a weak antigen, by being attached to a stronger antigen as carrier (i.e. a heterologous protein), becomes more immunogenic than if it were presented alone. If an animal has been previously immunized with the carrier alone, the animal may be "primed" and produce an enhanced immune response not only to carrier antigen but also to attached hapten groups. Infants are routinely immunized with tetanus and diphtheria toxoids. Thus, they would be primed for subsequent presentation of a capsular polymer antigen conjugated to either of these toxoids.

In general, any heterologous protein could serve as a carrier antigen. However, certain bacterial toxins such as tetanus and diphtheria may have an additional advantage in that they are composed of two portions, one of which (the "binding" subunit) has a strong affinity for binding to mammalian cell surfaces. Conceivably, conjugation to such a "binding" protein would permit the carried antigen to more effectively initiate responses in cells of the immune system.

The carrier proteins to which the capsular polymer is conjugated may be native toxin or detoxified toxin (toxoid). Also, by relatively recent mutational techniques, one may produce genetically altered proteins which are antigenically similar to the toxin yet non-toxic. These are called "cross reacting materials", or CRMs. CRM.sub.197 is noteworthy since it has a single amino acid change from the native diphtheria toxin and is immunologically indistinguishable from it.

Conjugation of capsular polymer to native toxin may reduce toxicity, but significant toxicity may remain. Thus, further detoxification of protein toxins employs formalin, which reacts with free amino groups of the protein. Residual toxicity may still be a concern. Furthermore, spontaneous detoxification is possible with any particular lot of vaccine and remains an issue of concern with this approach.

Alternatively, native toxin may be detoxified with formalin to produce conventional toxoid before conjugation to capsular polymer. However, the prior formalin treatment reduces the number of free amino groups available for reaction with the reducing groups of the capsular polymer fragment. CRMs, thus, have significant advantages in that they have no inherent toxicity yet none of their amino groups are occupied by the formalin. A further advantage is that no biohazards exist in working with CRMs.

In the case of CRM.sub.197, which is immunologically identical to native toxin, treatment with formalin (though there is no need to detoxify) greatly enhances the immunological response. It is thought that this is due to stabilization of the molecule against degradation by mechanisms of the body and/or aggregation by cross-linking (immunogenicity of particles increases with size).

For all of the above reasons, tetanus and diphtheria toxins are prime candidates for carrier proteins, yet there are others which may also be suitable. Though these others may not have the history of safety found with diphtheria and tetanus, there may be other overwhelming reasons to use them. For instance, they may be even more effective as carriers, or production economics may be significant. Other candidates for carriers include toxins of pseudomonas, staphylococcus, streptococcus, pertussis and Escherichia coli.

In a specific embodiment of the invention, activated oligosaccharides may be linked to CRM.sub.197 protein which has been purified as follows:

CRM.sub.197, produced by the strain Corynebacterium diphtheriae, may be separated from culture

medium by passing the bacterial culture through a Millipore membrane, precipitating protein from the filtrate, and purifying CRM.sub.197 by ion exchange chromatography, as described in section 6, infra. Alternatively, substantially pure CRM.sub.197 may be obtained by any method known in the art.

Activated oligosaccharide may be covalently linked to carrier protein in the presence of an organic solvent and, optionally, any other agent (such as a condensing agent) in order to promote the linkage of the terminal functional group of the activated oligosaccharide to the protein. In a specific, preferred embodiment of the invention, activated oligosaccharide bearing a terminal ester group may be covalently linked to free amino groups present on carrier protein as follows:

Activated oligosaccharide may be dissolved in dimethylsulfoxide and then added to an aqueous solution of carrier protein (for example, but not limited to CRM.sub.197 at a concentration of about 2 mg/ml) such that the molar ratio of monoester-activated oligosaccharide/total amino groups of the carrier protein is about 1:2 and the final concentration of DMSO is about 50% v/v. The conjugation reaction is performed 4.degree. C. and although the reaction is near to completion in about 2 hours, it is suitable to leave the reaction going overnight in order to increase the yield of reaction at the highest values for each type specific glycoconjugate. The glycoconjugates so obtained are then purified by gel chromatography.

For the synthesis of a monovalent vaccine, oligosaccharides derived from a single serotype of bacterium may be conjugated to protein. For the synthesis of a multivalent vaccine, glycoconjugates may be produced by 2 a mixture of oligosaccharides derived from bacteria of different species or different serotypes to a carrier protein; alternatively, glycoconjugates produced by reacting a single type of oligosaccharide with carrier protein in separate reactions using different oligosaccharides, may be mixed. Thus, a multivalent vaccine may comprise a carrier protein bearing a homogeneous or a heterogeneous population of linked oligosaccharides.

5.4. Immunochemical Characterization of Glycoconjugates

Verification of the immunogenicity of the glycoconjugates produced by the above method may be tested in any suitable animal system prior to administration to humans, including, but not limited to rabbits, pigs, guinea pigs, mice, rats, or goats. In a specific embodiment of the invention, rabbits (approximately 2 kg in weight) may be inoculated subcutaneously with glycoproteinic conjugate in the presence or absence of aluminum phosphate or hydroxide. Approximately 2.5 .mu.g of oligosaccharide would constitute an appropriate dose for a 2 kg rabbit. Antibody titers may then be evaluated by enzyme-linked immunosorbent assay (ELISA) or any other method known in the art. Since the antibodies generated toward the glycoconjugates of the invention may be incapable of imaunoprecipitating antigen, antibody assays dependent upon immunoprecipitation are not recommended for determining titers.

5.5. Vaccine Formulation and Administration

Suitable carrier media for formulating a vaccine include sodium phosphate-buffered saline (pH 7.4) or 0.125M aluminum phosphate gel suspended in sodium phosphate-buffered saline at pH 6 and other conventional media.

Generally, vaccines containing from about 5 to about 100 .mu.g, preferably about 10 to 50 .mu.g of oligosaccharide, are suitable to elicit effective levels of antibody against the capsular polymer in young warm-blooded mammals. Of course, the exact dosage would be determined by routine dose/response experimentation. The concentration of the glycoproteinic conjugates for the preparation of vaccines for children is comprised within the range of about 25 to 200 .mu.g of oligosaccharide. Greater doses may be administered on the basis of body weight. Several small doses given sequentially would be expected to be

superior to the same amount of conjugate given as a single injection.

The vaccines of the invention may be administered to warm-blooded mammals of any age and are especially adapted to induce active immunization against systemic infections in young mammals caused by the pathogens Haemophilus influenzae type b, Escherichia coli, Streptococcus pneumoniae, Neisseria meningitidis, and Pseudomonas aerugenosa.

According to the invention, vaccine may be delivered subcutaneously, intravenously, intramuscularly, intraperitoneally, orally, or intranasally. Vaccine may comprise glycoconjugate in soluble or microparticular form, or incorporated into microspheres or microvesicles, including liposomes.

5.6. Utility of Oligosaccharide Conjugate Vaccines

In preferred embodiments of the invention, glycoconjugate vaccines directed against encapsulated pathogenic bacteria are used to protect susceptible individuals from developing infections caused by these agents. Susceptible individuals include young children with immature immune systems, asplenic individuals, as well any individual with a compromised immune system or chronic disease, particularly acquired immunodeficiency syndrome (A.I.D.S.), hematopoietic malignancy, diabetes, chronic heart disease, chronic pulmonary disease, and sickle cell anemia. The glycoconjugates of the invention, by virtue of their conjugation to a carrier protein, enhance the immunogenicity of the oligosaccharides they carry.

Thus, the glycoconjugates of the invention may be used in vaccinations to confer protection against infection with any bacteria which possesses a polysaccharide capsule, including Streptococcus pneumoniae, Haemophilus influenzae, Escherichia coli, Neisseria meningitidis, Salmonella typhi, Streptococcus mutans, Cryptococcus neoformans, Klebsiella pneumoniae, Staphylococcus aureus, and Pseudomonas aerugenosa. Strains of S. pneumoniae particularly virulent in children, and specifically provided for by the present invention, include types 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, IIA, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23, and 33F.

In particular embodiments, the vaccines of the invention may be used to induce a monospecific and homogeneous immune response. A monospecific immune response is associated with a number of advantages, including providing antibodies with (i) homogeneous specificity, in which substantially all antibodies are directed against a specific epitope and are characterized by the same affinity constant value; (ii) a high affinity constant value with superior anti-bacterial activity; (iii) increased target specificity and absence of cross-reactivity with host related antigens, resulting in a safer vaccine; and (iv) decreased complement activation due to decreased precipitating activity monospecific antibodies, also resulting in a safer vaccine.

In additional embodiments, the present invention may be used to produce vaccines which recognize peptides or lipooligosaccharide or other surface oligosaccharide haptens linked, by methods of the invention, to carrier proteins. Such vaccines may be used, for example, in the induction of immunity toward tumor cells, or in the production of anti-tumor antibodies conjugated to a chemotherapeutic or bioactive agent; such anti-tumor activity could be induced by a tumor-specific antigen, or epitope thereof, to a carrier protein using methods of the invention.

6. EXAMPLE: DEVELOPMENT OF A MULTIVALENT PNEUMOCOCCAL OLIGOSACCHARIDE CONJUGATE VACCINE

6.1. Preparation of Polysaccharide

S. pneumoniae type 6A capsular polysaccharide, S. pneumoniae type 14 capsular polysaccharide, S. pneumoniae type 19F capsular polysaccharide, and S. pneumoniae type 23F capsular polysaccharide were obtained from the American Type Culture Collection.

6.2. Hydrolysis of Polysaccharide

6.2.1. Hydrolysis of S. Pneumoniae Type 6A Polysaccharide

Two milligrams of Type 6A S. pneumoniae capsular polysaccharide were dissolved in 1 ml. of aqueous solution containing 10 mM of acetic acid at pH =3.4, then allowed to hydrolyze in sealed ampules dipped in an oil bath at a temperature of 100.degree. C. for thirty hours. The resulting oligosaccharides were then separated from the reaction mixture by chromatography over Sephadex G15 (Pharmacia, Uppsala) conditioned with a 15 mM solution of NaCl at ph 7.0 at 4.degree. C.

The chromatographic effluents were then analyzed according to the procedures as reported by Kabat, (1964 in "Experimental Immunochemistry", Ed. E. A. Rabat and Mayer, pp. 538-541), Chen et al. (1956, Anal. Chem. 28:1756-1758), and Porro et al. (1981, Anal. Biochem. 118:301-306) to establish the presence of methyl-pentoses, phosphorous, and reducing groups, e.g. aldehyde groups. Analysis revealed a methyl pentose/aldehyde ratio of 3.96, a methyl pentose/phosphorous ratio of 0.96, and a phosphorous/aldehyde ratio of 4.12.

Gel chromatography on Sephadex G-50 Superfine (Pharmacia) using buffer, revealed a distribution constant (kd) of 0.538 (by hexose), corresponding to a molecular weight of approximately 2,500.

N.M.R., gas chromatography and stoichiometric analysis indicated that the oligosaccharides consisted of about 3-4 basic repeating units among which galactose, which was the immunodominant sugar, was found.

6.2.2. Hydrolysis of S. Pneumoniae Type 14 Polysaccharide

Two milligrams of Type 14 S. pneumoniae capsular polysaccharide was dissolved in 1 ml. of aqueous solution containing 0.5 M trifluoroacetic acid, then allowed to hydrolyze in sealed ampules dipped in an oil bath at a temperature of 70.degree. C. for seven hours. The resulting oligosaccharides were then separated from the reaction mixture by chromatography over Sephadex G15 (Pharmacia, Uppsala) conditioned with a 15 mM solution of NaCl at pH 7.0 at 4.degree. C.

The chromatographic effluents were then analyzed for hexosamine and aldehyde content and found to have a hexosamine to aldehyde ratio of 3.17. Gas chromatography and stoichiometric analysis indicated a molecular size corresponding to three to four basic repeating units. Debranching of galactose, as determined by gas chromatography, was within 10%. Gel chromatography on Sephadex G-50 Superfine (Pharmacia) using 15 mM NaCl at pH 7.0, revealed, for the oligosaccharide, a distribution constant (Kd) of 0.30 as determined by total hexose.

6.2.3. Hydrolysis of S. Pneumoniae Type 19F Polysaccharide

Two milligrams of Type 19F S. pneumoniae capsular polysaccharide were dissolved in 1 ml. of aqueous solution containing 10 mM of acetic acid at pH =3.4, then allowed to hydrolyze in sealed ampules dipped in an oil bath at a temperature of 50.degree. C. for forty-eight hours. The resulting oligosaccharides were then separated from the reaction mixture by chromatography over Sephadex G-15 (Pharmacia, Uppsala) conditioned with a 15 mM solution of NaCl at pH =7.0 at 4.degree. C.

The chromatographic effluents were then analyzed according to the procedures as reported by Kabat (1964, in "Experimental Immunochemistry," Ed. E. A. Rabat and Mayer, pp. 538-541), Chen et al. (1956, Anal. Chem. 28:1756-1758), and Porro et al. (1981, Anal. Biochem. 118:301-306) to establish the presence of methyl-pentoses, phosphorous, and reducing groups, e.g. aldehyde groups. Analysis revealed a methyl pentose/reduced methyl pentose ratio of 3.5, and a methyl pentose/phosphorous ratio of 3.2.

Gel chromatography on Sephadex G-50 Superfine (Pharmacia) revealed for the oligosaccharide a Kd =0.46 (by total hexose) and combined analysis by gas chromatography and stoichiometry indicated a size corresponding to three to four basic repeating units.

6.2.4. Hydrolysis of S. Pneumoniae type 23F Polysaccharide

Two milligrams of Type 23F S. pneumoniae capsular polysaccharide were dissolved in 1 ml. of aqueous solution of 0.25 M trifluoroacetic acid, then allowed to hydrolyze in sealed ampules dipped in an oil bath at a temperature of 70.degree. C. for three hours. The resulting oligosaccharides were then separated from the reaction mixture by chromatography over Sephadex G15 (Pharmacia, Uppsala) conditioned with a 15 mM solution of NaCl at pH =7.0 at 4.degree. C.

The chromatographic effluents were then analyzed according to the procedures as reported by Kabat (1964, in Experimental Immunochemistry, Ed. E. A. Rabat and Mayer, pp. 538-541), Chen et al. (1956, Anal. Chem. 28:1756-1758), and Porro et al. (1981, Anal. Biochem. 118:301-306) to establish the presence of methyl pentoses, phosphorous, and reducing groups, e.g. aldehyde groups. Analysis revealed a hexose/aldehyde ratio of 4.5 -4.5, a hexose/methyl pentose ratio of 2.3, and a phosphorous/aldehyde ratio of 2.9.

Gas-chromatography and stoichiometric analyses indicated the presence of between 3.5 and 4.5 basic repeating units. Debranching of rhamnose, as determined by gas chromatography, was less than eight percent.

Gel chromatography on Sephadex G-50 Superfine (Pharmacia) revealed a distribution constant (Kd) of 0.38 (by hexose).

6.3 Immunochemical Characterization of S. Pneumoniae Oligosaccharide Haptens

The ability of S. pneumoniae type 6A, 14, 19F, and 23F oligosaccharides to interact with antibodies directed against intact capsular polysaccharides was tested as described in Porro et al. (1985, Mol. Immunol. 22:907-919), using a technique which measures the ability of a hapten (i.e. the oligosaccharide) to inhibit the homologous antigen (capsular polysaccharide) to antibody immunoprecipitation reaction (low molecular weight haptens do not give an immunoprecipitation reaction when tested toward homologous antibodies).

The method, termed "differential immunoelectrophoresis," was performed as follows: a plastic plate support for immunoelectrophoresis contained three 1% (w/v) agarose compartments (Agarose M-LKB, Bromma, Sweden). The first compartment contained 0.05% (v/v) of reference antiserum to capsular polysaccharide. The second compartment contained 0.05% (v/v) of reference antiserum to capsular polysaccharide which had previously been incubated with a known amount of reference capsular polysaccharide at 37.degree. C. for 15 minutes. The third compartment contained 0.05% (v/v) of reference antiserum to capsular polysaccharide which had previously been incubated with a known amount of oligosaccharide hapten. An electrophoretic separation of capsular polysaccharide in four serial two-fold dilutions was then performed at 70 V/cm in 20 mM Tris-barbiturate buffer, pH =8.8, for 90 minutes. After

electrophoresis, the plates were silver-stained, dried, and quantified. Inhibition by the oligosaccharide molecules was evidenced by higher "rocket" immunoprecipitates appearing in the compartment containing the reference antiserum pre-incubated with hapten. The minimal inhibitory concentration of a hapten was calculated as ##EQU1## where C.sub.Ha =concentration of the hapten examined in the gel

h.sub.Ag =intercept of the straight line as determined by the height of the "rocket" immunoprecipitates obtained when the reference antigen was in the gel, and

h.sub.Ha =intercept of the straight line as determined by the height of the "rocket" immunoprecipitates obtained when the hapten examined was in the gel. Similarly

MIC.sub.Ag =C.sub.Ag .multidot.H.sub.AG

Specificity =MIC.sub.ag MIC.sub.Ha

Oligosaccharide haptens of different sizes were tested.

The ability of oligosaccharides to block immunoprecipitation of capsular polysaccharides by specific antibody was also tested by the nonelectrophoretic method of radial immunodiffusion. According to this method, inhibition by oligosaccharide molecules was evidenced by a larger radius of immunoprecipitate formed by diffusion of antigen (capsular polysaccharide) through 1 percent w/v agarose containing the specific antibody previously incubated with a given amount of inhibitor (oligosaccharide). Once the Minimal Combining Concentration (MCC) for the given hapten is experimentally found, specificity is then calculated according to the previously mentioned formula: ##EQU2##

TABLE III

Immunochemical Characterization of S. pneumoniae Oligosaccharide Haptens

Oligosaccharide type .sup.-- -DP .sup.-- --MW	(MIC.sub.Ps /MIC.sub.Hp) yby DIEP	(MCC.sub.Ps /MCC.sub.Hp) by IRID
6A	2 1.5K 10.sup.-3	
	3.5 2.5K 10.sup.-3	10.sup.-3
	10 7.0K 10.sup.-1	
14	5 3.5K n.t.	10.sup.-1
	15 10.4K	
	n.t.	10.sup.-1
19F	3.5 2.2K 10.sup.-3	10.sup.-4
23F CH.sub.3 COOH (hyd)		
	3 2.3K 10.sup.-3	10.sup.-2
	6 4.5K 10.sup.-1	10.sup.-1
TFA (hyd)	4.5 3.4K 10.sup.-4	5 .times. 10.sup.-3
	9.5 7.2K 10.sup.-1	10.sup.-1

n.t. = not testable
DIEP = Differential Immunoelectrophoresis
IRID = Inhibition of Radical Immunodiffusion
MIC = Minimal Inhibitory Conc.
MCC = Minimal Combining Conc.

6.4. Activation of the End-Reducing Unit of S. Pneumonia Oligosaccharides

Oligosaccharide haptens, obtained as described in section 6.2, supra, were dissolved in water to a final concentration of about 5 mg/ml. To each solution, 0.1 ml of 0.2 M KH.sub.2 PO.sub.4 for each milliliter of solution volume was added and the pH raised to 9.2-9.4 by the required amount of diaminomethane (generally, a volume of 2 .mu.l of diaminomethane for each milliliter of solution is required). The mixture was maintained at 100.degree. C. for 15 minutes, upon which time an amount of about 4 .mu.l of pyridine borane for each milliliter of solution volume was added. The pH was adjusted at 9.2 by lN NaOH. The mixture was then transferred, in a sealed ampule, to an oil bath at 50.degree. C. for the next 48 hours. After that, the amino-activated oligosaccharide solution was neutralized by 1N HCl and purified on Sephadex G-15 Superfine (15 mM NaCI, pH

were pooled 7.01. The collected chromatographic fractions and freeze dried. Then, the freeze-dried residue was dissolved at 1? mg/ml in DMSO and added to a molar amount of SIDEA (or SIDES) corresponding to a 5:1 mol/mol ratio with respect to the amount of amino groups present in the freeze-dried compound. The reaction proceeded at room temperature for 4 hours and then, was added to the solution 4 volumes of 1.4 dioxane (final conc. 80% in 1,4 dioxane) in order to precipitate the ester activated oligosaccharide. The precipitate, collected by centrifugation, was washed three times with 1,4 dioxane and kept at -20.degree. C. or lower unless used in the conjugation process. The yield of the activation process for each of the four oligosaccharides is shown in Table IV.

TABLE IV

S. pneumoniae Oligosaccharide Activation:
Yield of Process (% w/w)

Serotype	Oligo-NH(CH.sub.2).sub.2 NH.sub.2	Oligo-NH(CH.sub.2).sub.2 NH-monoester	Overall
6A	75	93	70
14	73	90	66
19F	100	100	100
23F	50	90	45
Xg (.+-.s.d.)	74.5 (.+-.20)	93.3 (.+-.4.7)	70 (+23)

6.5. Conjugation of Activated Oligosaccharide to CRM.sub.197 Protein

6.5.1 Preparation of CRM.sub.197 Protein

CRM.sub.197, produced by Cornebacterium diphtheriae C7 (B.sup.tx-197), was separated from culture medium by molecular filtration using a Millipore XM-50 (NMWL 5.times.10.sup.-4) membrane. The protein was then precipitated by adding to the filtrate a saturated solution of ammonium sulfate (up to 65% w/v). Precipitated protein was collected by centrifugation, and redissolved in 0.01 M phosphate buffer (pH =7.2).

Further purification of CRM.sub.197 was achieved by ion-exchange chromatography using a 2.5.times.100 cm DEAE - Sepharose 6B/CL column (Pharmacia, Uppsala) conditioned in 0.01 M phosphate buffer at pH 7.2, using 0.09 M NaCl in 0.01 M phosphate buffer as eluent.

SDS polyacrylamide gel electrophoresis under reducing conditions (Pappenheimer et al., 1972, Immunochem. 9:891-906) indicated that 80% of the CRM.sub.197 obtained was in its native molecular form. The purity of the protein was found to be approximately 400 flocculation limit (Lf) per milligram.

6.5.2. Conjugation of Activated Oligosaccharides

The conjugation procedure consisted of coupling; the monosuccinimidyl ester-activated oligosaccharide haptens to the epsilon-amino group of the lysine residues of the carrier protein CRM.sub.197.

Dimethyl sulfoxide containing monosuccinimidyl ester (of adipic acid) activated oligosaccharides of S. pneumoniae type 6A, 14, 19F, and 23F capsular polysaccharides was then added to a 0.1 M bicarbonate solution pH=8.0 containing 2 mg/ml of CRM.sub.197 to produce a solution which was 50% in water and in which the molar ratio of ester-activated oligosaccharide to total amino groups of the carrier protein is 1:2.

The mixture so obtained was kept, under mild stirring, at 4.degree. C. for 15 hours. Oligosaccharides from each of the four serotypes were conjugated to protein in separate reactions. A summary of the physiochemical characterization of the glycoconjugates obtained is presented in Table V.

TABLE V

Glycoconjugate Characterization					
Serotype	.sup.-- -DP oligo	.sup.-- --MW oligo (SDS-PAGE)	SD .sup.-- --MW conjugate (Moles oligo/ Mole Prot.	% (w/w) Conj. Prot.	
6A	3	2.1K	77.6K	7	100
14	5	3.5K	85.1K	6	100
19F	3	1.9K	69.2K	4	100
23F	6	4.5K	85.0K	5	100

6.5.2.1. Comparison of the Efficiency of Conjugation Using as Linker the Succinimidyl Diester of Adipic Acid Versus the Succinimidyl Diester of Succinic Acid

The activated oligosaccharides formed by reaction with the succinimidyl diester of succinic acid (SIDES) were of the structure ##STR1## whereas those formed by reaction with the succinimidyl diester of adipic acid (SIDEA) were of the structure ##STR2## and thereby produce linkers of different sizes between oligosaccharide and conjugated protein (see FIG. 2). The efficiency of conjugation using SIDES versus SIDEA activated oligosaccharides was evaluated. As shown in FIG. 3A, B and C, only when the linker was derived from SIDEA did the protein appear to be in fully glycosylated form (where little or no free band of CRM.sub.197 was detectable).

6.6. Immunogenicity of S. Pneumoniae Glycoconjugates

Several formulations of the four glycoconjugate antigens were prepared and tested in rabbits (according to the schedule delineated in Table VI): type - specific glycoconjugates in monovalent formulation (2.5 or 5.0

.mu.g oligosaccharide per dose) or multivalent formulation (2.5 .mu.g of each oligosaccharide per dose) with and without aluminum hydroxide [Al(OH.sub.3]as mineral adjuvant (only in the multivalent formulation at 1 mg per dose were administered). Complete absorption of the four glycoconjugates to Al(OH).sub.3 occurred under the adopted conditions, since processing the supernatant of the multivalent formulation by either SDS-polyacrylamide gel electrophoresis or rocket immunoelectrophoresis did not reveal any detectable amount of free protein. An average dose of each glycoconjugate contained approximately 2.5 .mu.g oligosaccharide and 13 .mu.g of carrier protein CRM.sub.197 (comparable to the average human vaccination dose of diphtheria toxoid). The immunization schedule included a priming dose and two booster doses four weeks apart. Bleedings were performed at week 0, 4, 6, and 10.

TABLE VI

Immunization Schedule for Rabbits And Mice And Doses of Vaccines Immunization at week 0, 4, 8 Bleeding at week 0, 4, 6, 10
A. Soluble monovalent (single type) formulation
1 dose (0.5 ml): 2.5 .mu.g oligosaccharide and 13 .mu.g (5 Lf) CRM.sub.197
1 dose (0.5 ml): 5.0 .mu.g oligosaccharide and 26 .mu.g (10 Lf) CRM.sub.197
B. Soluble Polyvalent (mixed 4 types) formulation
1 dose (0.5 ml): 2.5 .mu.g type-specific oligo (Tot = 10 .mu.g oligos) and a total of 52 .mu.g (20 Lf) CRM.sub.197
C. Al(OH).sub.3 -ads Polyvalent (mixed 4 types) formulation
1 dose (0.5 ml): 2.5 .mu.g type-specific oligo (Tot = 10 .mu.g oligos) and a total of 52 .mu.g (20 Lf) CRM.sub.197 with 1 mg of Al(OH).sub.3

Table VII shows the RIA (FARR method) estimated amount of type-specific antibodies as well as the number of responders over the number of animals immunized. The ratio (R) indicates the fold increase reached after each immunizing dose.

Table VIII shows the ELISA titers in terms of IgG isotype Ab as well as the number of responders versus the number of animals immunized. The ratios -R.sub.1 -R.sub.2 -R.sub.3 indicate the fold increase in the titers after each immunizing dose, while the ratios R.sub.1, -R.sub.2, -R.sub.3, indicate the fold increase in the titers for a given immunizing dose in respect to the pre-titer. Table IX reports the qualitative results in terms of functionality of the induced IgG antibodies in the recognition of the polysaccharide capsule on living streptococci (Quellung reaction or Neufeld test).

Table X shows the diphtheria toxin-neutralizing titers induced in rabbits by the carrier protein CRM.sub.197, as estimated by Vero cells assay. Since a reference FDA antiserum was used as control, titers expressed in .mu./ml have also been included.

TABLE VII

RIA-estimated titers* of rabbits immunized** with oligosaccharides**** of . Pneumoniae

type 6A, 14, 19F, 23F covalently bound to the carrier protein CRM.sub.197
****
Soluble Form AL(OH).sub.3 -ads Form

week 0	week 4	week 6	week 11	week 0	week 4	week 6	week 11
type 6A							
n.d.	n.d.	150(2/6)	495(6/6)	n.d.	538(5/5)	3,190(5/5)	4,064(5/5)
						R = 6.0	R = 1.3
type 14							
n.d.	n.d.	230(1/6)	195(2/6)	n.d.	77(3/6)	203(4/5)	216(5/5)
						R = 2.6	R = 1.1
type 19F							
n.d.	n.d.	n.d.	75(6/6)	n.d.	72(6/6)	108(5/5)	188(5/5)
						R = 1.5	R = 1.7
type 23F							
n.d.	n.d.	400(1/6)	140(1/5)	n.d.	283(3/6)	n.d.	246(5/5)

*Data expressed as geometric mean of titers in ngN.sub.Ab /ml. Responders vs. total animals immunized are in parenthesis.
**Multivalent formulations of the four glycoconjugates in soluble and Al(OH)3 adsorbed (1 mg/dose form). Each glycoconjugate contained an average of 2.5 .mu.g of oligosaccharide and an average of 13 .mu.g of protein CRM.sub.197. Immunization at week 1, 4 and 9. Bleeding at week 0, 4, 6 and 11.
***type 6A and 19F oligosaccharides had an average .sup.-- -DP = 3
type 14 and 23F oligosaccharides had an average .sup.-- -DP = 5
****Glycoconjugate of type 6A had an average substitution degree (.sup.--SD) of oligosaccharides per unit of carrier protein (mol/mol) equal to 7.
SD for type 14 glycoconjugate was 6; for type 19F was 4 and for type 23F

was 5.

TABLE VIII

ELISA Results of the IgG isotype Ab titers* induced by a multivalent vaccine including the glycoconjugates of S. pneumoniae .sup.-- -DP = 3 + 6 capsular oligosaccharides type 6A, 14, 19F, 23F adsorbed to the mineral adjuvant Al(OH).sub.3

Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 11)
type 6A	<50 (0/5)	4,800 (5/5)	51,200 (5/5)
	R.sub.1 > 96.0	R.sub.2 = 10.7 (.alpha. < 0.01)	R.sub.3 = 2.5 (.alpha. < 0.01)
		R.sub.2 ' > 1,027	R.sub.3 ' > 2,600
			130,000 (5/5)
type 14	<50 (0.5)	360 (5/5)	4,480 (5/5)
	R.sub.1 > 7.2	R.sub.2 = 12.4 (.alpha. < 0.01)	R.sub.3 = 4.4 (.alpha. < 0.01)
		R.sub.2 ' > 89.3	R.sub.3 ' > 396.0
			19,000 (5/5)
type 19F	< 50 (0/5)	2,080 (5/5)	18,560 (5/5)
	R.sub.1 > 41.6	R.sub.2 = 9.0 (.alpha. < 0.01)	R.sub.3 = 1.9 (.alpha. < 0.01)
		R.sub.2 ' > 371.2	R.sub.3 ' > 704.0
			35,200 (5/5)
type 23F	<50 (0/5)	880 (5/5)	1,280 (5/5)
	R.sub.1 > 17.6	R.sub.2 = 1.5 (.alpha. < 0.01)	R.sub.3 = 9.3 (.alpha. < 0.01)
		R.sub.2 ' > 25.6	R.sub.3 ' > 237.6
			11,880**

*Titers expressed as geometric mean of the reciprocal of the highest serum dilution showing ABS value twice of the reaction background. In parenthesis is reported the number of animals (responders over total injected).

**The value involves the titer of an unusually high responder rabbit. Discarding two out of five immunized rabbits, the best and worst responder, here are the results of the remaining 3 rabbits for the serotype 23F:

(week 0)	(week 4)	(week 6)	(week 11)
<50 (0/5)	667 (3/3)	1,333 (3/3)	2,667 (3/3)
R.sub.1 > 13.3	R.sub.2 = 2.0 (.alpha. < 0.01)	R.sub.3 = 2.0 (.alpha. < 0.01)	
	R.sub.2 ' > 26.7	R.sub.3 ' > 53.3	

TABLE IX

Immunological Functionality of Rabbit Serum Ab to .sup.-- -DP = 3-6 Oligo-conjugated to CRM.sub.197 Qualitative Analysis

(Quellung Reaction* for Capsular Recognition)

Type 6A S. pneumoniae: Positive Reaction
Type 14 S. pneumoniae: Positive Reaction
Type 19F S. pneumoniae: Positive Reaction
Type 23F S. pneumoniae: Positive Reaction

*Performed according to the method of Austrian (1976), Mt. Sinai J. Med. 43: 699-709.

TABLE X

Antidiphtheria titers* using vero cells assay induced in rabbits immunized by the multivalent glycoconjugates synthesized with oligosaccharides of S. pneumoniae convalently linked to the carrier protein CRM.sub.197

Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 11)
Soluble form			
<10	<10	25 (0.019 .mu./ml	1,920 (1.4 .mu./ml
	R = 2.5	R = 77.0	
Al(OH).sub.3 -ads			
<10	20 (0.015 .mu./ml)	1,280 (0.96 .mu./ml)	3,840 (2.9 .mu./ml)
	R = 64.0	R = 3.0	

FDA ref. antiserum contained 6 .mu./ml and gave 50% protection at dil. 1/8,000.

*Titers expressed as reciprocal of the dilution to which the pool of antisera showed 50% protection to the cells, as estimated by .sup.3 HLeucine incorporation after exposure of the cells to diphtheria toxin. Numbers in parenthesis indicate the titers in .mu./ml as determined using the FDA reference antiserum as control.

6.7. Oligosaccharides of Chain Length DP=10-20 are Suboptimally Immunogenic

Two groups of glycoconjugate vaccines were synthesized according to the scheme of synthesis described supra but using saccharides of type 6A, 14, 19F, and 23F S. pneumoniae with two different "range-values" of chain length, namely DP =3-5 and DP=10-20. The question then became whether an oligosaccharide with a chain length of DP=20 or larger would also be the optimal immunogen (upon conjugation to the selected carrier protein CRM.sub.197) in terms of priming and boosting capability as compared to a much shorter chain length, such as a DP=3 oligosaccharide.

Rabbits were immunized using the protocol outlined in Table XI. As shown by comparing results presented in Tables XII and XIII, which relate to ELISA results of IgG isotype antibody titers induced by soluble S. pneumoniae oligosaccharides with DP=10-14 and DP=3-6, respectively, as well as those presented in Tables XIV and XV, which relate to ELISA results of IgG isotype antibody titers induce by S. pneumoniae

oligosaccharides with DP=10-14 and DP=3-6, respectively, adsorbed to Al(OH).sub.3, a DP=10-14 was not associated with enhanced immunogenicity. In fact, the IgG priming and boosting activities of DP=3-5 oligosaccharide conjugates were far greater than activities observed using DP =10-14 oligosaccharide conjugates. Not casually, all four carbohydrate structures investigated were associated with similar results. Further, neutralization of diphtheria toxin by glycoconjugates with DP=10-14 was found to be less effective than that achieved using glycoconjugates with DP=3-6 (Table XVI). Thus oligosaccharides of chain length DP= 10-20 are functional in conjugates of the present invention though oligosaccharides of DP=3-6 elicit higher titers of antibody.

TABLE XI

Immunization Schedule for Rabbits
The models of glycoconjugates were injected at a dose of 2.5 .mu.g carbohydrate. Since the models tested different only in the chain length of the covalently linked oligosaccharides, the corresponding amount of carrier protein was:

Base of Carbohydrate (.mu.g)	Dose of Protein Carrier (.mu.g)	Weigh Ratio (ww)
.sup.-- -DP = 3-6		
2.5	12.5	0.2
oligo-CRM.sub.197		
.sup.-- -DP = 10-14		
2.5	2.5	1.0
olido-CRM.sub.197		

Immunization at weeks 0, 4 and 8.
Bleeding at weeks 0, 4 and 10.

TABLE XII

ELISA Results of the IgG Isotype Ab Titers Induced by a Multivalent Vaccine Including the Glycoconjugates of S. pneumoniae .sup.-- -DP = 10-14 Capsular Oligosaccharides type 6A, 14, 19F, 23F in Soluble Form

Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 10)
type 6A			
<100	<100	<100	500 (2/5)
type 14			
<100	300	2,400 (3/5)	4,600 (3/5)
type 19F			
<100	<100	<100	<100
type 23F			
<100	<100	<100	<100

TABLE XIII

ELISA Results of the IgG isotype Ab titers Induced by a Multivalent Vaccine Including the Glycoconjugates of S. pneumoniae .sup.-- -DP = 3-6 Capsular Oligosaccharides type 6A, 14, 19F, 23F in soluble form

Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 11)
Type			
<50	<200	967 (6/6)	8,500 (6/6)

6A R.sub.3 = 8.8 (.alpha. < 0.01)
Type <50 1,800 3,266 (3/6) 3,650 (4/6)
14
Type <50 <50 675 (4/6) 1,750 (6/6)
19F
Type <50 <50 <50 <50
23F

TABLE XIV

ELISA Results of the IgG Isotype Ab Titers Induced by a Multivalent Vaccine Including the Glycoconjugates of S. pneumoniae .sup.-- -DP = 10-14 Capsular Oligosaccharides type 6A, 14, 19F, 23F Adsorbed to the Mineral Adjuvant Al(OH).sub.3

Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 10)
Type	<100	240 (5/5)	900 (5/5)
			500 (5/5)
6A	R.sub.1 > 2.4		
	R.sub.2 = 3.8 (.alpha. < 0.01)		
	R.sub.2 ' > 9.0		
Type	<100	300 (5/5)	1,040 (5/5)
			8,480 (5/5)
14	R.sub.1 > 3.0		
	R.sub.2 = 3.5 (.alpha. < 0.01)		
		R.sub.3 = 8.2 (.alpha. < 0.01)	
	R.sub.2 ' > 10.4		
		R.sub.3 ' > 84.9	
Type	<100	<100	400 (1/5)
			800 (1/5)
19F			
Type	<100	<100	< 100 200 (1/5)
23F			

TABLE XV

Table IV. ELISA Results of the IgG isotype AB titers* induced by a multivalent vaccine including the glycoconjugates of S. pneumoniae .sup.-- -DP = 3-6 capsular oligosaccharides type 6A, 14, 19F, 23F adsorbed to the mineral adjuvant Al(OH).sub.3

Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 11)
Type	<50 (0/5)	4,800 (5/5)	
		51,200 (5/5)	
			130,000 (5/5)
6A	R.sub.1 > 96.0		
	R.sub.2 = 10.7 (.alpha. < 0.01)		
		R.sub.3 = 2.5 (.alpha. < 0.01)	
	R.sub.2 ' > 1,027		
		R.sub.3 ' > 2,600	
Type	<50 (0/5)	360 (5/5)	
		4,480 (5/5)	
			19,800 (5/5)
14	R.sub.1 > 7.2		
	R.sub.2 = 12.4 (.alpha. < 0.01)		
		R.sub.3 = 4.4 (.alpha. < 0.01)	
	R.sub.2 ' > 89.3		
		R.sub.3 ' > 396.0	

Type	<50 (0/5)	2,080 (5/5)		
			18,560 (5/5)	
				35,200 (5/5)
19F	R.sub.1 > 41.6			
	R.sub.2 = 9.0 (.alpha. < 0.01)			
			R.sub.3 = 1.9 (.alpha. < 0.01)	
	R.sub.2 ' > 371.2			
			R.sub.3 ' > 704.0	
Type	<50 (0/5)	880 (5/5)		
			1,280 (5/5)	
				11,880 (5/5)
23F	R.sub.1 > 17.6			
	R.sub.2 = 1.5 (.alpha. < 0.01)			
			R.sub.3 = 9.3 (.alpha. < 0.01)	
	R.sub.2 ' > 25.6			
			R.sub.3 ' > 237.6	

*Titers expressed as geometric mean of the reciprocal of the highest seru dilution showing ABS value twice of the reaction background. In parenthesis is reported the number of animals (responders over total injected).

TABLE XVI

In vitro Neutralization* of Diphtheria Toxin with Sera from Rabbits Immunized with S. pneumonia Oligosaccharide-CRM.sub.197 Glycoconjugates

	Pre-titer (week 0)	Priming (week 4)	1st Booster (week 6)	2nd Booster (week 10)
.sup.-- -DP = 3-6 oligo-CRM197:				
Soluble	<1/10	<1/10	1.20 (0.03 .phi./ml)	1/1,280 (2.05 .phi./ml)
Al(OH).sub.3 ads				
	<1/10	1/10 (0.016 .phi./ml)	1/640 (1.02 .phi./ml)	1/2,560 (4.10 .phi./ml)
.sup.-- -DP = 10-14 oligo-CRM197:				
Soluble	<1/10	<1/10	<1/10	1/10 (0.106 .phi./ml)
Al(OH).sub.3 ads				
	<1/10	<1/10	1/40 (0.06 .phi./ml)	1/80 (0.13 .phi./ml)

*Titers expressed as reciprocal of the dilution of which the pool pf rabbit antisera showed 50% protection to the cells, as estimated by Hleucine incorporation after exposure of the cells to diptheria toxin. Numbers in parenthesis indicate the titers in .mu.g/ml as determined by the FDA reference antiserum as control.
MPL estimated in humans: 0.01 .mu.g/ml.

6.8. The Immune Response to the Glycoconjugates is Monospecific and Homogeneous

Comparison of the results depicted in Tables VII and VIII, which relate to antibody titers determined by radioimmunoassay (RIA) and enzyme linked immunosorbant assay (ELISA), reveals that the RIA

estimated titers were consistently lower than ELISA-estimated titers. This observation, together with the absence of immunoprecipitates in agarose gels used for radial immunodiffusion and rocket electrophoresis analysis of anti-glycoconjugate antiserum, proves that the rabbit antisera to S. pneumoniae oligosaccharide-CRM.sub.197 conjugates contained highly specific IgG isotype antibodies which were unable to precipitate the respective purified carbohydrate polymers used to generate the oligosaccharides.

The absence of precipitating antibodies in an antiserum is indicative of monospecificity, i.e., antibody recognition of only one epitope in the antigenic repertoire Immunol. 18:751-763). Precipitation of antigen-antibody complexes requires lattice formation to generate a three-dimensional, branching network of linked antigen and antibody molecules. For this to occur, multivalency of both antigen and antibody is required, as more than one antibody must be able to bind to a single antigen molecule simultaneously. Thus, the lack of observable immunoprecipitation occurring between rabbit antiserum to S. pneumoniae oligosaccharide-CRM.sub.197 conjugates and homologus purified high molecular the antisera contained antibodies specific for the carbohydrate polymer (as shown by ELISA and inhibition-ELISA analyses) but directed toward only one determinant (epitope) of the polysaccharide.

In addition to exhibiting immunoprecipitating activity, a heterogeneous population of antibodies is also generally associated with the following property; a single epitope of the antigen used to elicit the antibody response cannot completely inhibit the binding of the entire population of antibodies to complete antigen, but will only inhibit those antibodies binding to that one epitope, leaving the other antibodies free to bind to the remaining epitopes present on complete antigen. A population of antibodies may be evaluated for heterogeneity by an ELISA-inhibition assay. In this assay, the ability of a population of antibodies to bind to complete antigen can be measured in the presence of inhibitors of antigen/antibody binding, such as isolated epitopes of the antigen. Represented graphically when the binding of antibody to labeled completed antigen is measured in the presence of increasing concentrations of unlabeled complete antigen, a sigmoidal curve is generated, which can be used as a standard curve for antibody/antigen binding. If the antibody population is heterogeneous, binding between antibody and complete antigen cannot be completely inhibited by the addition of a single antigenic epitope, and the standard curve of antibody/antigen binding is only partly displaced (partly overlapped or partly paralleled) as other antigen-antibody interactions, distinct from those associated with the epitope being tested, predominate. Conversely, binding of a homogeneous population of antibodies to antigen can be completely inhibited by the addition of an isolated epitope; the standard sigmoidal antigen/antibody binding curve for a homogeneous population of antibodies will be overlapped or paralleled by the curve generated by the addition of isolated epitope corresponding to the population's specificity.

Experimentally, by testing the S. pneumoniae glycoconjugate induced rabbit IgG in this manner, an affinity pattern was observed corresponding to that predicted for a homogeneous population of antibodies (FIG. 4). The S. pneumoniae 6A oligosaccharide, (either in non-conjugated or conjugated form), was associated with binding inhibition sigmoidal curve approximately parallel to one derived using serotype 6A high molecular weight capsular polysaccharide. As expected, a heterologous (type 14) oligosaccharide, in either free (linker-activated) or conjugated form, did not inhibit the IgG isotype population specific for the type 6A antigen.

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=> s neisseria meningitidis

         14536 NEISSERIA
            11 NEISSERIAS
           188 NEISSERIAE
         14598 NEISSERIA
                 (NEISSERIA OR NEISSERIAS OR NEISSERIAE)
          5548 MENINGITIDIS
L1        5481 NEISSERIA MENINGITIDIS
                 (NEISSERIA(W)MENINGITIDIS)

=> s neisseria meningitidis b

         14536 NEISSERIA
            11 NEISSERIAS
           188 NEISSERIAE
         14598 NEISSERIA
                 (NEISSERIA OR NEISSERIAS OR NEISSERIAE)
          5548 MENINGITIDIS
        599127 B
L2          36 NEISSERIA MENINGITIDIS B
                 (NEISSERIA(W)MENINGITIDIS(W)B)

=> s neisseria meningitidis c

         14536 NEISSERIA
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         14598 NEISSERIA
                 (NEISSERIA OR NEISSERIAS OR NEISSERIAE)
          5548 MENINGITIDIS
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L3          10 NEISSERIA MENINGITIDIS C
                 (NEISSERIA(W)MENINGITIDIS(W)C)

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            11 NEISSERIAS
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         14598 NEISSERIA
                 (NEISSERIA OR NEISSERIAS OR NEISSERIAE)
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        937761 C
            10 NEISSERIA MENINGITIDIS C
                 (NEISSERIA(W)MENINGITIDIS(W)C)
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AN   1995:221217  BIOSIS
DN   PREV199598235517
TI   Evaluation of the effectiveness of targeted vaccination with
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     polysaccharide vaccine A+C in a locality in the Czech Republic.
AU   Krizova, P. (1); Vlckova, J.; Bobak, M.
CS   (1) NRL pro meningokokove nakazy, CEM-SZU, Srobarova 48, 100 42 Praha 10
     Czech Republic
SO   Epidemiologie Mikrobiologie Imunologie, (1995) Vol. 44, No. 1, pp. 9-14.
     ISSN: 1210-7913.
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SL   Czech; English


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DN   PREV200200304029
TI   Analysis of the ontogeny of the murine humoral response to
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     polysaccharide reveals levels of complexity relevant to vaccine
     development.
AU   Colino, Jesus (1); Outschoorn, Ingrid
CS   (1) Dept. of Pathology, Uniformed Services University of Health Sciences,
     4301 Jones Bridge Rd., Room B3073, Bethesda, MD, 20814: jcolino@usuhs.mil
     USA
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     ISSN: 0022-1899.
DT   Article
LA   English

L2   ANSWER 2 OF 36  BIOSIS  COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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AN 2002:198459 BIOSIS
DN PREV200200198459
TI Cancer vaccines: An update with special focus on ganglioside antigens (Review.
AU Bitton, Roberto J.; Guthmann, Marcelo D.; Gabri, Mariano R.; Carnero, Ariel J. L.; Alonso, Daniel F.; Fainboim, Leonardo; Gomez, Daniel E. (1)
CS (1) Molecular Oncology Laboratory, Department of Science and Technology, Quilmes National University, R. Saenz Pena 180, Bernal, 1876, Buenos Aires: degomez@unq.edu.ar Argentina
SO Oncology Reports, (March April, 2002) Vol. 9, No. 2, pp. 267-276. print. ISSN: 1021-335X.
DT General Review
LA English

L2 ANSWER 3 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2002:62183 BIOSIS
DN PREV200200062183
TI Coconjugates of OMPC, HIV related peptides and anionic moieties.
AU Emini, E. A.; Leanza, W. J.; Marburg, S.; Tolman, R. L.
CS Paoli, Pa. USA
ASSIGNEE: MERCK & CO., INC.
PI US 5606030 Feb. 25, 1997
SO Official Gazette of the United States Patent and Trademark Office Patents,
(Feb. 25, 1997) Vol. 1195, No. 4, pp. 2587-2589.
ISSN: 0098-1133.
DT Patent
LA English

L2 ANSWER 4 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2002:60875 BIOSIS
DN PREV200200060875
TI Method of producing **Neisseria meningitidis B** vaccine, and vaccine produced by method.
AU Huergo, C. C.; Sierra, Gonzalez, V. G.; Gutierrez, Vazquez, M. M.; Bisset,
Jorrin, G.; Garcia, Imia, L. G.; De, La, Caridad, Puentes, Rizo, G.; Del, Carmen, Sampedro, Herrera, M.; Sotolongo, Padron, F.; Le, Riverend, Morales, E. X.; Galguera, Dominguez, M. A.
CS Havana Cuba
ASSIGNEE: CENTRO NACIONAL DE BIOPREPARADOS
PI US 5597572 Jan. 28, 1997
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DT Patent
LA English

L2 ANSWER 5 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2001:417967 BIOSIS
DN PREV200100417967
TI Characteristics of Neisseria meningitidis strains isolated from invasive cases in Poland in 1995-2000.
Original Title: Charakterystyka szczepow Neisseria meningitidis izolowanych od osob z objawowym zakazeniem meningokokowym w latach 1995-2000 w Polsce..
AU Grzybowska, W. (1); Tyski, S. (1)
CS (1) Zaklad Antybiotykow i Mikrobiologii Instytutu Lekow, ul. Chelmska 30/34, 00-725, Warszawa Poland
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ISSN: 0025-8601.
DT Article
LA Polish
SL English; Polish

L2 ANSWER 6 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2001:260975 BIOSIS
DN PREV200100260975
TI System for the expression of heterologous antigens as fusion proteins.
AU Cano, Carlos Antonio Durate (1); Nieto, Enrique Gerardo Guillen; Acosta, Anabel Alvarez; Munoz, Luis Emilio Carpio; Vazquez, Diogenes Quintana; Rodriquez, Carmen Elena Gomez; de la Caridid Siva Rodriguez, Recardo; Galvez, Consuelo Nazabal; Angulo, Maria De Jesus Leal; Dunn, Alejandro Miguel Martin
CS (1) Habana Cuba
ASSIGNEE: Centro de Ingenieria Genetica Y Biotecnologia, Havana, Cuba
PI US 6146635 November 14, 2000
SO Official Gazette of the United States Patent and Trademark Office Patents,
(Nov. 14, 2000) Vol. 1240, No. 2, pp. No Pagination. e-file.
ISSN: 0098-1133.
DT Patent
LA English

L2 ANSWER 7 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2000:516931 BIOSIS
DN PREV200000516931
TI Surveillance of cases of meningococcal disease associated with military recruits studied for meningococcal carriage.
AU Andersen, Jesper (1); Berthelsen, Lene; Jensen, Bente Bech; Lind, Inga
CS (1) Neisseria Department, Statens Serum Institut, Artillevivej 5, DK-2300,
Copenhagen S Denmark
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ISSN: 0036-5548.
DT Article
LA English
SL English

L2 ANSWER 8 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2000:480881 BIOSIS
DN PREV200000480881
TI Usefulness of pulsed-field gel electrophoresis in assessing clonal relationships between **Neisseria meningitidis B** :14:P1-7,16 from an endemic area in France.
AU Nouvellon, M. (1); Barbier-Frebourg, N. (1); Guibourdenche, M.; Riou, J. Y.; Berthelot, G.; Lemeland, J. F.
CS (1) Chu de Rouen, Rouen France
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Meeting Info.: 39th Interscience Conference on Antimicrobial Agents and Chemotherapy San Francisco, California, USA September 26-29, 1999 American
Society for Microbiology
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DT Conference
LA English
SL English

L2 ANSWER 9 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 2000:226708 BIOSIS
DN PREV200000226708
TI The siaA gene involved in capsule polysaccharide biosynthesis of **Neisseria meningitidis B** codes for N-acylglucosamine-6-phosphate 2-epimerase activity.
AU Petersen, Michael; Fessner, Wolf-Dieter; Frosch, Matthias; Lueneberg, Edeltraud (1)
CS (1) Institut fuer Hygiene und Mikrobiologie, Universitaet Wuerzberg, Josef-Schneider-Str. 2, D-97080, Wuerzburg Germany

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ISSN: 0378-1097.
DT Article
LA English
SL English

L2 ANSWER 10 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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AU De Gaspari, E. N. (1); Belo, E.F.T. (1); Coutinho, L.M.C. (1)
CS (1) Secao de Imunologia, Instituto Adolfo Lutz, Sao Paulo, SP Brazil
SO Immunology Letters, (June 15, 1999) Vol. 69, No. 1, pp. 155.
Meeting Info.: 10th International Congress of Mucosal Imunology Amsterdam,
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ISSN: 0165-2478.
DT Conference
LA English

L2 ANSWER 11 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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DN PREV199900014123
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AU Dagan, Ron (1); Fraser, Drora; Roitman, Malvina; Slater, Paul; Anis, Emilia; Ashkenazi, Shai; Kassis, Imad; Miron, Dan; Leventhal, Alexander; Bacteremia, The Israeli Pediatric; Group, Meningitis
CS (1) Pediatric Infectious Disease Unit, Soroka Univ. Med. Cent. Ben-Gurion Univ. Negev, Beer-Sheva Israel
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ISSN: 0264-410X.
DT Article
LA English

L2 ANSWER 12 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1998:416001 BIOSIS
DN PREV199800416001
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AU Milagres, L. G. (1); Gorla, M. C. O.; Barroso, D. E.
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Meeting Info.: 98th General Meeting of the American Society for Microbiology Atlanta, Georgia, USA May 17-21, 1998 American Society for Microbiology
. ISSN: 1060-2011.
DT Conference
LA English

L2 ANSWER 13 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1997:282631 BIOSIS
DN PREV199799581834
TI Serotypes and subtypes of **Neisseria meningitidis** **B** isolated from CSF.
AU Altschuler, M.; Gonzalez Ayala, S.; Regueira, M.; Agosti, M.; Palmerio, S.
CS Hosp. Ninos Sor Maria Ludovica, 14 No. 1631, 1900-La Plata Argentina
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Meeting Info.: 97th General Meeting of the American Society for Microbiology Miami Beach, Florida, USA May 4-8, 1997
ISSN: 1060-2011.
DT Conference; Abstract; Conference
LA English

L2 ANSWER 14 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1996:509605 BIOSIS
DN PREV199699231961
TI Quantitation of IgG subclass antibody responses after immunization with a group B meningococcal outer membrane vesicle vaccine, using monoclonal mouse-human chimeric antibodies as standards.
AU Naess, L. Meyer; Rosenqvist, E.; Hoiby, E. A.; Michaelsen, T. E. (1)
CS (1) Dep. Vaccinology, National Inst. Public Health, P.O. Box 4404, Torshov, Oslo N-0403 Norway
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ISSN: 0022-1759.
DT Article
LA English

L2 ANSWER 15 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1996:438609 BIOSIS
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AU Carroll, James A.; Dorward, David W.; Gherardini, Frank C. (1)
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DT Article
LA English

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AN 1996:380862 BIOSIS
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AU Zorgani, A. A.; James, V. S.; Stewart, J.; Blackwell, C. C. (1); Elton, R.
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CS (1) Dep. Med. Microbiol., Univ. Edinburgh, Teviot Place, Edinburgh EH8 9AG
UK
SO FEMS Immunology and Medical Microbiology, (1996) Vol. 14, No. 2-3, pp. 73-81.
ISSN: 0928-8244.
DT Article
LA English

L2 ANSWER 17 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1996:23041 BIOSIS
DN PREV199698595176
TI Expression of iron-regulated outer membrane protein in Neisseria meningitidis: A comparison of three culture media.
AU Jessouroun, Ellen (1); Danelli, Maria Das Gracas Miranda; De Almeida, Andre Luiz
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DT Article
LA English

L2 ANSWER 18 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

AN 1996:21810 BIOSIS
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TI The antibody response to a prototype liposome vaccine containing Neisseria meningitidis outer membrane protein P1 produced in Bacillus subtilis.
AU Idanpaan-Heikkila, Ilona (1); Muttilainen, Susanna; Wahlstrom, Eva; Saarinen, Leena; Leinonen, Maija; Sarvas, Matti; Makela, P. Helena
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ISSN: 0264-410X.
DT Article
LA English

L2 ANSWER 19 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1995:221217 BIOSIS
DN PREV199598235517
TI Evaluation of the effectiveness of targeted vaccination with meningococcal polysaccharide vaccine A+C in a locality in the Czech Republic.
AU Krizova, P. (1); Vlckova, J.; Bobak, M.
CS (1) NRL pro meningokokove nakazy, CEM-SZU, Srobarova 48, 100 42 Praha 10 Czech Republic
SO Epidemiologie Mikrobiologie Imunologie, (1995) Vol. 44, No. 1, pp. 9-14.
ISSN: 1210-7913.
DT Article
LA Czech
SL Czech; English

L2 ANSWER 20 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1995:109762 BIOSIS
DN PREV199598124062
TI The use of filter paper monoclonal antibodies in a Dot-blot test for typing **Neisseria meningitidis B**.
AU De Gaspari, E. N. (1); Ribeiro-Filho, A. A.; Zollinger, W. D.
CS (1) Secao Imunologia, Inst. Adolfo Lutz, Av. Dr. Arnaldo 355, 11 andar, 01246-902 Sao Paulo, SP Brazil
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ISSN: 0100-879X.
DT Article
LA English

L2 ANSWER 21 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1995:107036 BIOSIS
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AU Vazquez, Javier E. (1); Ayala, Marta (1); Danielsson, Lena; Fernandez De Cossio, Maria E. (1); Cruz, Silian (1); Nazabal, Consuelo; Mussachio, Alexis; Silva, Ricardo; Borrebaeck, Carl A. K.; Zollinger, Wendell D.; Gavilondo, Jorge V. (1)
CS (1) Div. Immunotechnol. Diagnostics, P.O. Box 6162, La Habana Cuba
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DT Article
LA English
SL English; Spanish

L2 ANSWER 22 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1994:531459 BIOSIS
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TI Molecular analysis of the biosynthesis pathway of the alpha-2,8 polysialic acid capsule by Neisseria meningitidis serogroup B.

AU Edwards, Ulrike; Mueller, Astric; Hammerschmidt, Sven; Gerardy-Schahn, Rita; Frosch, Matthias (1)
CS (1) Inst. Med. Mikrobiologie, Medizinische Hochschule Hannover, 30623 Hannover Germany
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DT Article
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L2 ANSWER 23 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1994:499583 BIOSIS
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CS (1) Dep. Med. Microbiol., Univ. Med. Sch., Teviot Place, Edinburgh EH8 9AG UK
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DT Article
LA English

L2 ANSWER 24 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1994:209944 BIOSIS
DN PREV199497222944
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AU Hammerschmidt, Sven; Birkholz, Carola; Zaehringer, Ulrich; Robertson, Brian D.; Van Putten, Jos; Ebeling, Olaf; Frosch, Matthias (1)
CS (1) Inst. Med. Mikrobiologie, Medizinische Hochschule Hannover, Konstanty Gutschow Strasse 8, 30623 Hannover Germany
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L2 ANSWER 25 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1993:252198 BIOSIS
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CS (1) Inmunologia, C.N.M.V.I.S., Instituto S. Carlos III, Majadahonda, 28220 Madrid Spain
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DT Article
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L2 ANSWER 26 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1992:523904 BIOSIS
DN BA94:131979
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CS DEP. MOL. BACTERIOL., NATIONAL PUBLIC HEALTH INST., MANNERHEIMINTIE 166, SF-0030 HELSINKI, FINLAND.
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L2 ANSWER 27 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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AU GUTTORMSEN H-K; BJERKNES R; NAESS A; LEHMANN V; HALSTENSEN A; SORNES S; SOLBERG C O
CS MED. DEP. B, UNIV. BERGEN, HAUKELAND HOSPITAL, N-5021 BERGEN, NORW.
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CODEN: INFIBR. ISSN: 0019-9567.
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L2 ANSWER 28 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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L2 ANSWER 29 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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AU MCGUINNESS B T; CLARKE I N; LAMBDEN P R; BARLOW A K; POOLMAN J T; JONES D M; HECKELS J E
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CODEN: LANAAI.
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L2 ANSWER 30 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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CODEN: ATMPA2. ISSN: 0003-4983.
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L2 ANSWER 31 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1988:497061 BIOSIS
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CS MERCK SHARP DOHME RES. LAB., WEST POINT, PA. 19486.
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FS BR; OLD
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L2 ANSWER 32 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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L2 ANSWER 33 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1987:106648 BIOSIS
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AU HARTHUG S; ROSENQVIST E; HOIBY E A; GEDDE-DAHL T W; FROHOLM L O
CS MED. DEP., N-5016 HAUKELAND HOSP., BERGEN, NORWAY.
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AN 1987:8509 BIOSIS
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L2 ANSWER 35 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1986:221305 BIOSIS
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CS HYBRIDOMA SECTION, LAB. CENTRE FOR DISEASE CONTROL, HEALTH AND WELFARE CANADA, OTTAWA, ONT., CANADA K1A 0L2.
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CODEN: CJMIAZ. ISSN: 0008-4166.
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LA English

L2 ANSWER 36 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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CODEN: BOMEBE. ISSN: 0021-7867.
FS BA; OLD
LA French

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     (FILE 'HOME' ENTERED AT 10:52:16 ON 11 JUN 2002)

     FILE 'BIOSIS' ENTERED AT 10:52:24 ON 11 JUN 2002
L1         5481 S NEISSERIA MENINGITIDIS
L2           36 S NEISSERIA MENINGITIDIS B
L3           10 S NEISSERIA MENINGITIDIS C
L4            0 S NEISSERIA MENINGITIDIS C AND NEISSERIA MENINITISDIS B
L5            1 S L2 AND L3

=> d l3


L3   ANSWER 1 OF 10  BIOSIS  COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN   2001:34220  BIOSIS
DN   PREV200100034220
TI   Investigation for a more virulent variant among the C:2b:P1.2,5 Spanish
     meningococcal epidemic strains by molecular epidemiology.
AU   Arreaza, L.; Berron, S.; Fernandez, S.; Santiago, M. I.; Malvar, A.;
     Vazquez, J. A. (1)
CS   (1) Laboratorio de Referencia de Meningococos, Servicio de Bacteriologia,
     Centro Nacional de Microbiologia, Instituto de Salud Carlos III, 28220,
     Majadahonda, Madrid: jvazquez@isciii.es Spain
SO   Journal of Medical Microbiology, (December, 2000) Vol. 49, No. 12, pp.
     1079-1084. print.
     ISSN: 0022-2615.
DT   Article
LA   English
SL   English


=> d l3 1-10


L3   ANSWER 1 OF 10  BIOSIS  COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN   2001:34220  BIOSIS
DN   PREV200100034220
TI   Investigation for a more virulent variant among the C:2b:P1.2,5 Spanish
     meningococcal epidemic strains by molecular epidemiology.
AU   Arreaza, L.; Berron, S.; Fernandez, S.; Santiago, M. I.; Malvar, A.;
     Vazquez, J. A. (1)
CS   (1) Laboratorio de Referencia de Meningococos, Servicio de Bacteriologia,
     Centro Nacional de Microbiologia, Instituto de Salud Carlos III, 28220,
     Majadahonda, Madrid: jvazquez@isciii.es Spain
SO   Journal of Medical Microbiology, (December, 2000) Vol. 49, No. 12, pp.
     1079-1084. print.
     ISSN: 0022-2615.
DT   Article
LA   English
SL   English

L3   ANSWER 2 OF 10  BIOSIS  COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN   1999:302250  BIOSIS
DN   PREV199900302250
TI   Unusual isolation of Neisseria meningitidis.
AU   Blahova, M. (1); Petrovicova, A.
CS   (1) Oddelenie klinickej mikrobiologie NsP, Sladkovicova 9, 965 01, Ziar
     nad Hronom Slovakia
SO   Epidemiologie Mikrobiologie Imunologie, (1999) Vol. 48, No. 2, pp.
60-62.
     ISSN: 1210-7913.
DT   Article
LA   Slovak
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SL English; Slovak

L3 ANSWER 3 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1999:147757 BIOSIS
DN PREV199900147757
TI Human antibody responses to A and C capsular polysaccharides, IgA1 protease and transferrin-binding protein complex stimulated by infection with Neisseria meningitidis of subgroup IV-1 or ET-37 complex.
AU Brieske, Norbert; Schenker, Martin; Schnibbe, Thomas; Quentin-Millet, Marie-Jose; Achtman, Mark (1)
CS (1) Max-Planck-Inst. fuer Mol. Genetik, Ihnestrasse 73, D-14195 Berlin Germany
SO Vaccine, (Feb., 1999) Vol. 17, No. 7-8, pp. 731-744.
ISSN: 0264-410X.
DT Article
LA English

L3 ANSWER 4 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1999:44245 BIOSIS
DN PREV199900044245
TI Factors influencing carriership of Neisseria meningitidis and Neisseria lactamica.
AU Krizova, P. (1); Vlckova, J.
CS (1) CEM, Statni zdravotni ustav, Srobarova 48, 100 42 Praha 10 Czech Republic
SO Epidemiologie Mikrobiologie Imunologie, (1998) Vol. 47, No. 4, pp. 131-136.
ISSN: 1210-7913.
DT Article
LA Czech
SL Czech; English

L3 ANSWER 5 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1998:79104 BIOSIS
DN PREV199800079104
TI Changes of clinical and epidemiological characteristics of meningococcal invasive disease in western Bohemia in conjunction with the incidence of an invasive clonus of Neisseria meningitidis.
AU Struncova, V. (1); Pazdiora, P.; Valchova, M.; Sedlacek, D.; Franova, D.; Barta, R.; Musilek, M.; Krizova, P.
CS (1) Infekcni Klin. FN, tr. E. Benese 13, 305 99 Plzen Czech Republic
SO Epidemiologie Mikrobiologie Imunologie, (1997) Vol. 46, No. 4, pp. 145-148.
ISSN: 1210-7913.
DT Article
LA Czech
SL Czech; English

L3 ANSWER 6 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1996:131037 BIOSIS
DN PREV199698703172
TI Targeted vaccination with meningococcal polysaccharide vaccine in one district of the Czech Republic.
AU Kriz, P. (1); Vlckova, J.; Bobak, M.
CS (1) Natl. Reference Lab. Meningococcal Infections, Natl. Inst. Public Health, Srobarova 48, 100 42 Prague 10 Czech Republic
SO Epidemiology and Infection, (1995) Vol. 115, No. 3, pp. 411-418.
ISSN: 0950-2688.
DT Article
LA English

L3 ANSWER 7 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1995:221217 BIOSIS
DN PREV199598235517
TI Evaluation of the effectiveness of targeted vaccination with meningococcal

polysaccharide vaccine A+C in a locality in the Czech Republic.
AU Krizova, P. (1); Vlckova, J.; Bobak, M.
CS (1) NRL pro meningokokove nakazy, CEM-SZU, Srobarova 48, 100 42 Praha 10 Czech Republic
SO Epidemiologie Mikrobiologie Imunologie, (1995) Vol. 44, No. 1, pp. 9-14. ISSN: 1210-7913.
DT Article
LA Czech
SL Czech; English

L3 ANSWER 8 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1995:85378 BIOSIS
DN PREV199598099678
TI New epidemiological situation in the Czech Republic caused by meningococcus C:2a:P1.2 (Pl.5.
AU Krizova, P. (1); Musilek, M.; Lebedova, V.
CS (1) Statni Zdravotni Ustav, Srobarova 48, 100 42 Praha 10 Czech Republic
SO Epidemiologie Mikrobiologie Imunologie, (1994) Vol. 43, No. 4, pp. 183-187.
ISSN: 1210-7913.
DT Article
LA Czech
SL Czech; English

L3 ANSWER 9 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1994:365280 BIOSIS
DN PREV199497378280
TI Characterization of epidemic Neisseria meningitidis serogroup C strains in
several Brazilian states.
AU Sacchi, Claudio Tavares (1); Tondella, Maria Lucia Cecconi; Lemos, Ana Paula Silva De; Gorla, Maria Cecilia Outeiro; Berto, Denise Bonato; Kumiochi, Nilce Haida; Melles, Carmo Elias Andrade
CS (1) Bacteriol. Div., Adolfo Lutz Inst., Av. Dr. Arnaldo, 351 Sao Paulo, 01246-902 Brazil
SO Journal of Clinical Microbiology, (1994) Vol. 32, No. 7, pp. 1783-1787. ISSN: 0095-1137.
DT Article
LA English

L3 ANSWER 10 OF 10 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AN 1994:134648 BIOSIS
DN PREV199497147648
TI Increased incidence of invasive meningococcal disease in some regions in the Czech Republic caused by meningococcus C:2a:P1.2.
AU Krizova-Kuzemenska, P. (1); Benes, C.; Dlhy, J.; Kartusek, S.; Matuska, J.; Vlckova, J.; Galetkova, A.; Roznovsky, L.
CS (1) Statni Zdravotni Ustav, Srobdrova 48, 100 42 Praha 10, CZE
SO Ceskoslovenska Epidemiologie Mikrobiologie Imunologie, (1993) Vol. 42, No.
4, pp. 165-171.
ISSN: 0009-0522.
DT Article
LA Czech
SL Czech; English

=> d his

(FILE 'HOME' ENTERED AT 10:52:16 ON 11 JUN 2002)

FILE 'BIOSIS' ENTERED AT 10:52:24 ON 11 JUN 2002
L1 5481 S NEISSERIA MENINGITIDIS
L2 36 S NEISSERIA MENINGITIDIS B
L3 10 S NEISSERIA MENINGITIDIS C

L4 0 S NEISSERIA MENINGITIDIS C AND NEISSERIA MENINITISDIS B
L5 1 S L2 AND L3

=> d l2 1-36 ab

L2 ANSWER 1 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Although purified capsular polysaccharide of Neisseria meningitidis group B (CpsB) is not immunogenic at any age, CpsB on the bacterial surface elicits antibody responses late in ontogeny. Therefore, a detailed analysis of the ontogeny of the murine anti-CpsB response to N. meningitidis could determine key parameters regarding the poor immunogenicity of CpsB. The effects of bacterial dose, hyperimmunization, age, and sex on the induction of primary and secondary anti-CpsB immunoglobulin isotype profiles were studied. It was demonstrated that the timing and repetition of immunization and of the bacterial dose have a marked differential effect on the primary induction of anti-CpsB immunoglobulin isotypes and on the ability to induce anti-CpsB antibody responses after subsequent rechallenge. It is noteworthy that the ontogeny of the response is related to the appearance of natural anti-CpsB antibodies, but this is not associated with the presence of CpsB cross-reactive antigens in the microflora.

L2 ANSWER 2 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Vaccine development is one of the most promising and exciting fields in cancer research; numerous approaches are being studied to developed effective cancer vaccines. The aim of this form of therapy is to teach the patient's immune system to recognize the antigens expressed in tumor cells, but not in normal tissue, to be able to destroy these abnormal cells leaving the normal cells intact. In other words, is an attempt to teach the immune system to recognize antigens that escaped the immunologic surveillance and are 'tolerated' by it, therefore able to survive and, in time, disseminate. However each research group developing a cancer vaccine, uses a different technology, targeting different antigens, combining different carriers and adjuvants, and using different immunization schedules. Most of the vaccines are still experimental and not approved by the US or European Regulatory Agencies. In this work, we will offer an update in the knowledge in cancer immunology and all the anticancer vaccine approaches, with special enphasis in ganglioside based vaccines. It has been demonstrated that quantitative and qualitative changes occur in ganglioside expression during the oncogenic transformation. Malignant transformation appears to activate enzymes associated with ganglioside glycosylation, resulting in altered patterns of ganglioside expression in tumors. Direct evidence of the importance of gangliosides as potential targets for active immunotherapy has been suggested by the observation that human monoclonal antibodies against these glycolipids induce shrinkage of human cutaneous melanoma metastasis. Thus, the cellular over-expression and shedding of gangliosides into the interstitial space may play a central role in cell growth regulation, immune tolerance and tumor-angiogenesis, therefore representing a new target for anticancer therapy. Since 1993 researchers at the University of Buenos Aires and the University of Quilmes (Argentina), have taken part in a project carried out by the 'Centro de Inmunologia Molecular' (CIM) from La Havana, Cuba, to developed new strategies for specific active immunotherapy. The project included two ganglioside based vaccines and one anti-idiotypic vaccine. We focused on two antigens: first GM3, an ubiquitous antigen which is over-expressed in several epithelial tumor types; and a second one, N-Glycolyl-GM3 a more 'tumor specific' molecule,

not being expressed in normal tissues and recently found in several neoplastic cells, in particular breast, melanoma and neuroectodermal cancer cells. We developed two vaccines, one with each antigen, both using proteins derived from the outer membrane proteins (OMP) of **Neisseria Meningitidis B**, as carriers. We developed also the 1E10 vaccine; an anti-idiotype vaccine designed to mimic the N-Glycolyl-GM3 gangliosides. This monoclonal antibody is an Ab2-type-antibody which recognizes the Ab1 antibody called P3, the latter is a monoclonal antibody that specifically recognizes 'N-Glycolylated' gangliosides as antigens. Since 1998 we initiated a clinical development program for these three compounds. Results of the phase I clinical trials proved that the three vaccines were safe and able to elicit specific antibody responses. In addition we were able to demonstrate the activation of the cellular arm of the immune response in patients treated with the GM3 vaccine. Although phase I trials are not designed to evaluate antitumor efficacy, it was encouraging to observe tumor shrinkage in some patients treated both with the GM3 and N-Glycolyl-GM3 vaccines. We have already begun a phase II program in several neoplastic diseases, with all three vaccines.

L2 ANSWER 3 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 4 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 5 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

AB Phenotype and genotype identification of 179 Neisseria meningitidis strains isolated from cerebrospinal fluid or blood of patients with meningococcal infection, hospitalized in Poland, was performed. This is the first analysis of that type conducted in Poland. We analyzed strains collected in 1995-2000 from laboratories located all over the country. Phenotype **Neisseria meningitidis B**:22:P1.14 was the predominant among analyzed invasive strains in Poland. Type 22 is characteristic to most of the strains isolated in our country. No strain from analyzed group belonged to known epidemic clusters. One penicillin resistant strain (MIC=2 mg/l) and about 27% strains with decreased susceptibility to penicillin (0.1=<MIC<1.0 mg/l) were present among 166 N. meningitidis tested. All strains were susceptible to ciprofloxacin and rifampicin.

L2 ANSWER 6 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

AB The present invention relates to biotechnology and genetic engineering, particularly the expression of proteins of viral origin in microorganisms through their fusion, by applying the recombinant DNA technology, to bacterial peptides. The present invention provides an efficient process for the expression in Escherichia coli of heterologous proteins as fusion polypeptides with a view to obtaining them with a high degree of purity, in commercially useful amounts, and in an appropriate form for their inclusion in vaccine preparations intended to human use. To this effect, what is essentially used is a stabilizing sequence derived from the first 47 amino acids of the antigen P64k of **Neisseria meningitidis B**:4:P1.15. In particular, use is made of a recombinant plasmid containing said sequence, under the control of the tryptophane promotor of E. coli and of the terminator of the transcription of the phage T4, including restriction sites which provide for the cloning in phase of DNA fragments coding for polypeptides of interest. The process of the invention is applicable to the pharmaceutical industry, for the development of diagnostic systems, vaccine preparations, and in any situation where it is required to obtain high amounts of heterologous proteins as fusion polypeptides in E. coli.

L2 ANSWER 7 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Through a 14-months extended surveillance of meningococcal disease in Denmark, all 322 notified cases were investigated for possible connection with a military camp where 3 cohorts of recruits (n = 1069) were studied prospectively for meningococcal carriage. One case occurred in a recruit who was a constant non-carrier during the preceding 3 months. The invasive **Neisseria meningitidis B**:1:P1.1,7 strain was isolated from the pharynx only in 3 out of 17 room-mates (18%); the strains were identical as assessed by genotyping (PFGE and ribotyping). Two civilian cases outside the camp had direct contact with 2 recruits, but neither these 2 nor other recruits in the relevant divisions carried the invasive strains on any occasion. Six civilian cases had marginal relationship with the camp, but no contact with the recruits. In conclusion, pheno- and genotyping concordantly demonstrated a high carriage rate of the invasive strain among the room-mates to a recruit with meningococcal disease. Transmission to the patient most likely occurred shortly before onset of illness. The extended surveillance did, however, not identify any unexpected epidemiological links and restriction of antibiotic chemoprophylaxis to household/sleeping/kissing contacts in sporadic cases of meningococcal disease seems appropriate and relevant.

L2 ANSWER 8 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 9 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB The capsule polysaccharide of Neisseria meningitidis serogroup B is composed of a homopolymer of alpha-2fwdarw8 linked N-acetyl-neuraminic acid (sialic acid). The enzymes required for sialic acid biosynthesis and polymerization are encoded in region A of the capsule gene complex. We here describe the enzymatic activity of the siaA gene product as determined by biochemical analysis. siaA was overexpressed in Escherichia coli and the SiaA protein was purified to homogeneity. Enzymatic assays revealed that SiaA did not accept N-acetyl-glucosamine as substrate, but only N-acetyl-glucosamine-6-phosphate (EC 5.1.3.9). SiaA catalyzes the isomerization of N-acetyl-glucosamine-6-phosphate to form N-acetyl-mannosamine-6-phosphate. This reaction represents the first step in capsule biosynthesis of N. meningitidis B.

L2 ANSWER 10 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 11 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB An ongoing nationwide prospective surveillance program for invasive H. influenzae b (Hib) disease in Israel enabled us to study the effectiveness of a national infant Hib immunization program, which included all infants born since January 1994. The vaccine used was Hib polysaccharide conjugated to outer membrane protein complex of **Neisseria meningitidis b** (PRP-OMPC). For the cohort born during the 3 years since January 1994, the vaccine effectiveness was 94.9% for all invasive Hib diseases and 96.6% for meningitis. The efficacy in fully immunized subjects was 98.7 and 99.5%, respectively. A herd immunity effect could be observed, since a reduction in cases also occurred among infants too young to be immunized. No increase in invasive cases caused by S. pneumoniae and N. meningitidis was observed during the study period. This is the first report outside North America and Western Europe that demonstrates a nationwide extensive reduction of invasive Hib disease within a short time of the introduction of Hib conjugate vaccines to the infant immunization program.

L2 ANSWER 12 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 13 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 14 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

AB An ELISA method was developed to quantitate gravimetrically (mu-g/ml) the IgG subclass response against a Norwegian vaccine composed of outer membrane vesicles (OMV) isolated from a **Neisseria meningitidis B**: 15:P1.7,16 epidemic strain. Chimeric mouse-human anti-hapten NIP (5-iodo-4-hydroxy-3-nitrophenacetyl) antibodies of each subclass were used for calibration purposes. Before vaccination, low amounts of IgG1 and IgG2 antibodies against OMV were detectable in all vaccinees, whereas IgG3 was only detectable in one of the 21 vaccinees. After vaccination, IgG1 antibodies dominated the response followed by IgG3 and low to moderate levels of IgG2 antibodies. IgG4 was only detectable at very low levels in a few vaccinees. All sera showed close to parallel dose-response curves to each other for IgG1 and IgG3, whereas the IgG2 curves were not parallel to chimeric IgG2 and could thus not be quantitated gravimetrically. For IgG3, 1/3 of the vaccinee sera showed non-parallel dose-response curves to the rest of the vaccinee sera and to chimeric IgG3 and could not be gravimetrically quantitated. The rest of the sera showed parallel dose-response curves with the chimeric IgG3 and gravimetric quantitation was possible. This study illustrates that chimeric antibodies can be used as calibrators to quantitate IgG subclass antibody responses against OMV in gravimetric units and that the vaccine mainly induces IgG1 and IgG3 antibodies in humans.

L2 ANSWER 15 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Bacterial pathogens have evolved various strategies to acquire iron from the iron-restricted environment found in mammalian hosts. Borrelia burgdorferi should be no different with regard to its requirement for ferric iron, and previous studies have suggested that transferrin (Tf) may be a source of iron in vivo. By probing blots with Tf conjugated to horseradish peroxidase, we have identified an outer membrane protein (28 kDa) from B. burgdorferi B31 that bound holo-Tf but not apo-Tf. The 28-kDa protein bound human, rat, or mouse Tf and was produced only by low-passage (less than passage 5), virulent isolates of strain B31. In addition, the Tf-binding protein (Tbp) from strain B31 retained the ability to bind Tf after treatment with 2% sodium dodecyl sulfate-1% beta-mercaptoethanol and heating to 100 degree C for 5 min. These properties are remarkably similar to those of the Tbp of Staphylococcus aureus and Tbp2 from **Neisseria meningitidis**. **B**. burgdorferi Sh-2-82 produced an outer membrane protein different in size, i.e., 26 kDa, but with properties similar to those of to the protein from strain B31, suggesting variation in B. burgdorferi Tbps. The exact role of the 28-kDa protein in iron acquisition by B. burgdorferi remains to be determined.

L2 ANSWER 16 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Sera obtained from 106 children following an outbreak of **Neisseria meningitidis** (**B**:4:P1.15) were screened for bactericidal antibodies against isolates of meningococci and Neisseria lactamica. Most had high titers of antibodies to N. lactamica and N. meningitidis NG:4:- but not to capsulate isolates: B:4:P1.15; B:15:P1.16; B:4:-; C:4:-. Bactericidal activity was higher for both carriers and secretors but the differences were not significant. Bactericidal activity was not associated with total or specific IgA or IgM. Carriers had significantly higher levels of IgG to N. lactamica but not to NG:4:- in sera with bactericidal activity for each of the capsulate strains. Among non-carriers, higher levels of IgG to N. lactamica were associated with killing of B:4:P1.15 and B:4:-. Secretors' sera with bactericidal activity had significantly higher levels of IgG to N. lactamica compared with sera that were not bactericidal. This was not observed among non-secretors. Antibodies to the

outbreak strain were adsorbed by all Neisseria isolates tested and absorption of sera with N. lactamica alone completely removed the bactericidal activity against the outbreak strain.

L2 ANSWER 17 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB SDS-PAGE followed by Coomassie blue staining was used to analyse the iron-regulated protein (IRP) expressed on the outer membrane of two strains of **Neisseria meningitidis B**:4:P1.15 (N44/89 and CU385). The organisms were cultured on three different media (TSB, Catlin and Franz) with and without iron-chelator. Under iron-starvation conditions the meningococci expressed new proteins on their outer membrane including proteins of 60-90 kD and a 37 kD protein. In Catlin and TSB media, there was no difference between the expressed proteins for the two strains. On the other hand, the protein outline was unchanged when the strains were cultured in Franz medium with and without EDDHA, with exception of a 55 kD protein induced by the N44/89 only in Franz with EDDHA. In addition, the protein profiles were different when compared with Catlin and TSB media. The IRP expressed by meningococci belonging to the same serotype and subtype varied qualitatively and quantitatively and the induction of these proteins depended on the medium formulation used.

L2 ANSWER 18 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Monoclonal antibodies to the class 1 outer membrane protein P1 of **Neisseria meningitidis B**:15:P1.7,16 have been shown to be bactericidal and protective in an infant rat meningitis modeL We have produced the P1 protein in Bacillus subtilis as inclusion bodies. When the purified and denatured protein (BacP1) was reconstituted with phosphatidylcholine into liposomes, native antigenic epitopes were formed.
Such liposomes were reproducibly immunogenic in mice and guinea pigs at a low dose (1-10 mu-g of BacP1 protein) and without any other adjuvant. The resulting antisera contained high titers (enzyme immunoassay) of antibodies directed to native P1 epitopes exposed on the surface of meningococcal cells. The sera were also active with live N. meningitidis in bactericidal assays and protective in the infant rat meningitis model; all these activities were specific to the serosubtype of the P1 protein.

L2 ANSWER 19 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB In the Czech Republic where meningococcal disease occurred only sporadically for a very long period and **Neisseria meningitidis B** prevailed, the use of a meningococcal polysaccharide vaccine was never indicated. This situation changed in 1993 when a new clone of Neisseria meningitidis C:2a:P1.2(P1.5) appeared in the
Czech Republic, found to be responsible for a new epidemiological and clinical situation. The disease caused by this new clone is more serious, showing a high fatality rate (20%) and frequently an atypical clinical course. In May 1993, the highest age-specific incidence in the most affected locality was established in the age group of 15-19 years (52.1/100 000), while in the whole Czech Republic the respective figure was 2.4. A vaccination campaign focusing on the most affected age group started in this locality at the beginning of June 1993, using a polysaccharide meningococcal vaccine A+C (Merieux). During two weeks 6191 students of the age group of 15-19 years were vaccinated, i.e. 96% of all students of this age group, 64.5% of the population 15-19 years old and 5.6% of the whole population of this locality. This age targeted vaccination prevented the spread of the meningococcal invasive disease caused by Neisseria meningitidis C in this locality. The decrease in morbidity in this locality is statistically highly significant (p lt 0.001). In another affected locality, where vaccination was not age targeted and showed a very low coverage, the incidence of the invasive disease caused by Neisseria meningitidis C did not decrease. During the following period (1993-1994) the new meningococcal clone spread to all regions of the Czech Republic. Active surveillance of meningococcal

invasive disease has been conducted with the aim to recognize as early as possible an emerging epidemiological indication for targeted vaccination.

L2 ANSWER 20 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB A simple method for the collection, preservation, shipment, and testing of minute amounts of dried monoclonal antibodies for typing **Neisseria meningitidis B** is described. The monoclonal antibodies collected on filter paper were extracted in PBS and evaluated by Dot-blot employing whole cells of N. meningitidis B as antigen. The dried filter paper with monoclonal antibodies could be stored at room temperature for as long as 30 days without detectable changes in antibody response when used for typing outer membrane antigens of N. meningitidis B.

L2 ANSWER 21 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Many monoclonal antibodies (MAbs) had been generated for classification purposes against the porin proteins of Neisseria meningitidis, a Gram negative bacteria responsible for meningococcal disease. These MAbs could also be potentially important for passive serotherapy, based on their ability to mediate complement-dependent cell lysis (CDC), block the adhesion of the bacteria to endothelia, or stimulate phagocytosis. MAbs 15-1-P4 and CB-Nm.2 are mouse IgG2b that identify the class 3 outer membrane protein of N. meningitidis, serogroup B. Our group has previously shown that these MAbs compete for a B:385 (B:4:P1.15) serotype antigen preparation with almost identical affinity constants. However, when confronted with a Neisseria panel using whole-cell ELISA, their strain recognition varies, strongly indicating that they react with spatially close but nevertheless different epitopes on class 3 OMP. In this paper we show that both MAbs have similar CDC ability for strain B:385. Due to their potential as agents for passive immunotherapy, we have cloned the variable regions of the antibodies using the polymerase chain reaction (PCR), and determined their sequence. We found that these belong to different mouse gene families, and show very low homology.

L2 ANSWER 22 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB The genes encoding all enzymes necessary for capsular polysaccharide biosynthesis in **Neisseria meningitidis B** are located on a 5 kb DNA fragment within the chromosomal cps gene cluster. Nucleotide sequence analysis revealed four open reading frames (ORFs), which can encode proteins with molecular masses of 41.4 kDa, 24.9 kDa, 38.3 kDa, and 54.4 kDa, respectively. These ORFs constitute a transcriptional unit as demonstrated by Northern blots. Primer extension analysis revealed that the transcriptional start site is preceded by a nucleotide sequence with homologies to the sigma-70 consensus promoter sequence of Escherichia coli. Functional analysis of the proteins encoded by the ORFs indicated that ORF2 encodes the CMP-NeuNAc synthetase, ORF3 encodes the NeuNAc condensing enzyme, and ORF4 encodes the alpha-2,8 polysialyltransferase. ORF1 encodes an enzyme, which provides a precursor molecule for synthesis of monomeric NeuNAc. In E. coli the subcloned ORFs 2-4 were able to synthesize a high-molecular-weight alpha-2,8 polysialic acid. In contrast, inactivation of ORF1 in the meningococcal genome resulted in a complete loss of capsule production. A regulatory enzyme, the CMP-NeuNAc hydrolase, which cleaves CMP-NeuNAc to CMP and NeuNAc, was not found as a part of the capsular polysaccharide biosynthesis gene operon or within the cps gene cluster.

L2 ANSWER 23 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Carriage of **Neisseria meningitidis B**:4:P1.15 was higher among non-secretors during a school outbreak of meningitis; non-secretors had lower levels of anti-meningococcal salivary IgM. Flow cytometry was used to assess effects of secretor and non-secretor saliva on binding of B:4:P1.15 to buccal epithelial cells: (1) to assess inhibition by IgA and IgM: and (2) to assess contributions of salivary antibodies to inhibitory activities. Greater inhibition was obtained with

secretor saliva: pooled (P = 0.049); fresh (P = 0.0001). Purified IgA (P = 0.02) and IgM (P = 0.03) were equally inhibitory. After absorption of anti-meningococcal antibodies, there was still significant inhibitory activity in the pools: secretors (P = 0.018); non-secretors (P = 0.005). These results indicate that both secretory immunoglobulins and other factors contribute to protection against colonization by meningococci and might explain the increased carriage of B:4:P1.15 in this population.

L2 ANSWER 24 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Within the capsule gene complex (cps) of **Neisseria meningitidis B** a 5.5 kb DNA fragment encodes proteins with strong homologies to enzymes of the lipopolysaccharide biosynthetic pathway of Salmonella typhimurium and Escherichia coli, GalE, RfbB, RfbC and RfbD. A meningococcal galE mutant expressed a truncated lipooligosaccharide (LOS), which terminated at the glucose residue between inner and outer core, and a second galE gene present outside the cps cluster was found to be transcriptionally and functionally inactive and, thus, unable to complement this defect. Because of the defect in the outer core, the LOS of the galE-defective meningococcal mutant was not sialylated. In contrast, carbohydrate analysis of the LOS of an rfb-defective meningococcal mutant revealed no difference from the LOS of the wild-type strain, suggesting that the rfb genes are inactive. This was supported by Northern blot analysis, which showed that expression of the rfb gene products was transcriptionally regulated. The inability of the meningococcal galE mutant, which cannot sialylate the LOS, allowed us to investigate the significance of LOS sialylation in relation to the presence of the polysialic acid capsule. Sialylated LOS, but not the polysialic acid capsule, is necessary to confer complete serum resistance on the meningococcus by inhibition of the alternative complement pathway.

L2 ANSWER 25 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB A method is described for the selective biotinylation of meningococcal capsular polysaccharide from Neisseria meningitidis group B and its application to an enzyme-linked immunoabsorbent assay (ELISA) to detect specific antibodies by immobilization on streptavidin-coated microtiter wells. Capsular polysaccharide from **Neisseria meningitidis B** has been biotinylated by specific periodate oxidation of terminal residues and condensation of the resulting aldehydes with biotin hydrazide, using a spin-column technique in the intermediate purification steps. The ELISA was optimized employing an extended reaction time between the label alkaline phosphatase and its most common substrate, p-nitrophenyl phosphate, together with evaluation of blocking agents to minimize non-specific binding. Specificity was demonstrated by a direct competitive enzyme immunoassay (EIA).

L2 ANSWER 26 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB The class 1 outer membrane protein of **Neisseria meningitidis B**:15:P1.7,16, was expressed in Bacillus subtilis in high yield as intracellular aggregates. These were easy to isolate and the protein (called BacP1) could be solubilized under denaturing conditions. Sera of mice immunized with thus-solubilized BacP1 contained high titres of antibodies that reacted with the class I protein of the meningococcal envelope in immunoblots but did not react with native meningococcal envelope in enzyme immunoassays (EIA) or with intact meningococci in bactericidal assays. However, when the BacP1 protein was complexed with heterologous (Salmonella) lipopolysaccharide, the ensuing sera reacted with meningococai envelope preparations in both EIA and immunoblots, showed subtype-specific bactericidal activity, and were protective in an infant rat meningitic model.

L2 ANSWER 27 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB We have examined the opsonic activity of sera from patients with **Neisseria meningitidis** (**B**:15:P1.16) infections against different meningococcal strains, using flow cytometry and luminol-enhanced chemiluminescence. A marked increase in the phagocytosis of ethanol-fixed meningococcal strains of different serogroups, serotypes, and serosubtypes was demonstrated in the presence of convalescence sera compared with acute sera. Convalescence sera also caused a significant increase of leukocyte oxidative metabolism during phagocytosis, as measured by luminol-enhanced chemiluminescence. The sera contained a broad range of opsonins cross-reacting with serogroup A, B, C, W-135, and Y meningococci of different serotypes and serosubtypes, indicating that the cross-reacting opsonins recognized surface epitopes other than those determined by current serotyping schemes.

L2 ANSWER 28 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Poly-.alpha.-2,8 N-acetylneuraminic acid (polySia) is an important virulence factor in infections caused by Escherichia coli K1 and **Neisseria meningitidis B**. In E. coli K1 a membranous CMP-NeuAc: poly-.alpha.-2,8 sialosyl sialyltransferase (polysialyltransferase) complex catalyses the synthesis of linear polySia chains. The complex also elongates sialyl oligomers that serve as exogenous acceptors. The gene encoding a polysialyltransferase of E. coli has been identified by subcloning and DNA sequence analysis. The subcloned DNA fragment codes for a polypeptide with a molecular mass of 47 kDa catalysing the in vitro synthesis of polySia by elongation of exogenous acceptors.

L2 ANSWER 29 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB The por A gene, which encodes expression of meningococcal class 1 outer membrane protein, responsible for antigenic subtype specificity, has been cloned and sequenced in an isolate of **Neisseria meningitidis** (**B**:15:P1.7,16) from a patient in the Gloucester area with meningococcal meningitis. Comparison of the sequence with that of the equivalent gene from the P1.7,16 reference strain reveals a point mutation which generates a single aminoacid change in the epitope responsible for P1.16 specificity. Monoclonal antibodies with P1.16 specificity do not react with synthetic peptides that correspond to the altered epitope, and do not promote complement-mediated bactericidal killing of the isolate. Analysis of other strains shows widespread distribution of infections due to B:15:P1.7,16 meningococci with the altered epitope (P1.16b) in England and Wales.

L2 ANSWER 30 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB A prospective study using a Latex particle agglutination test for the detection of bacterial antigens in CSF has been carried out in 91 patients in Kamuzu Central Hospital, Malawi. The antigens sought were those of Streptococcus pneumoniae, Haemophilus influenzae b, **Neisseria meningitidis B**/E. coli K1, and Neisseria meningitidis A,C,Y,W 135. Forty-one patients had proven bacterial meningitis, two had tuberculous meningitis, 39 had cerebral malaria, four had aseptic meningitis and five had convulsions. The sensitivity and specificity of the tests (Str. pneumoniae, 88% and 100%; H. influenzae b, 87% and 96%; N. meningitidis A,C,Y,W 135, 100% and 100%; and N. meningitidis B, 100% and 98%) were as good as those reported from developed countries. Unlike in some other parts of Africa, group B meningococci seem to predominate in cases of meningococcal meningitis in Malawi.

L2 ANSWER 31 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

L2 ANSWER 32 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB The protective efficacy of monoclonal antibodies to class 1 and class 3 outer membrane proteins of **Neisseria meningitidis** **B**:15:P1.16 was tested in an infant rat infection model. Four monoclonal antibodies to class 1 protein had bactericidal titres exceeding 20 000 and they protected infant rats completely against bacterial challenge with meningococci carrying the same class 1 protein, P1.16. One monoclonal antibody to class 3 protein was highly bactericidal (titer > 20 000), whereas two others had no bactericidal activity. All these antibodies gave some protection from infection, resulting in mortalities varying from 66 to 83% as compared to 100% in control rats who had received either unrelated monoclonal antibody or saline. These results strongly speak for class 1 outer membrane protein as a vaccine candidate for meningococcus group B.

L2 ANSWER 33 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB To elucidate pathogenic aspects and serodiagnostic possibilities for meningococcal disease, we investigated levels of specific antimeningococcal immunoglobulin G (IgG), IgA, and IgM in serum by using an enzyme-linked immunosorbent assay with outer membrane antigen prepared from a **Neisseria meningitidis** **B**:15:P1.16 strain. Serum samples were drawn on hospital admission as well as during convalescence from patients suspected of purulent meningitis or meningococcal septicemia, and single samples were drawn from population controls. A total of 637 samples were examined blindly. On admission, the average antimeningococcal immunoglobulin levels were about the same in the meningococcal disease patients as in the population controls. Septicemic patients, however, had significantly lower values. During one week the mean specific immunoglobulin levels in meningococcal-disease patients increased 6 times for IgG, 14 times for IgA, and 5 times for IgM. Children younger than 1 year showed a modest and more slowly developing antibody response. There were no statistically significant differences in average antibody responses among patients infected with meningococci of different serotypes. At 100% specificity, the increase in IgG, IgA, and IgM yielded diagnostic sensitivities for meningococcal disease of 84, 52, and 66%, respectively. One of seven serum pairs from the patient control group with unknown etiology was positive for meningococcal disease in this assay. The patients with meningococcal disease originally diagnosed with only by clinical signs and symptoms showed a slightly lower rate of seroconversion than the patients in whom the diagnosis was supported by test results showing a systemic Neisseria meningitidis isolate.

L2 ANSWER 34 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB Examination of the trends of meningococcal infection in Norway, Iceland, Faroe Islands, Denmark, England and Wales, and the Netherlands, has shown that **Neisseria meningitidis** **B**:2b:P1.2 and/or B:2a:P1.2 phenotypes were associated with peaks of infection in the Netherlands in 1966, in Iceland 1976-77, and in England and Wales in 1973-75. These strains were present in all six countries in the decade 1970-80 but their prevalence is now practically negligible. In contrast the prevalence of the B:15:P1.16 phenotype has risen. In the Faroe Islands and northern Norway this change in serotype prevalence has been followed by rises in incidence of meningococcal disease; the same is happening in England and Wales but not yet in the other countries.

L2 ANSWER 35 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
AB A disseminated and fatal infection was established in C57BL mice, injected

intraperitoneally with either **Neisseria meningitidis** **B**,2b or Haemophilus influenzae type b bacteria plus enhancement factors. The effects of mucin, hemoglobin, and iron dextran as enhancement of bacterial infectivity in mice were evaluated individually and in combination. A mixture of mucin and hemoglobin was most effective in enhancing the virulence of the pathogens. Inbred mouse lines were more susceptible than outbred ones. Relative virulence of a number of bacterial strains was also compared in one selected mouse line. **Neisseria** **meningitidis** **B**,2b and Haemophilus influenzae type b strains were more virulent than non-B,2b and nontypable strains. Finally, the course of bacteremia for the two infections in mice was followed by quantitative blood cultures. The animals succumbed to the generalized condition within 72 h. In the case of **Neisseria** **meningitidis** **B**,2b, 10 organisms with 4% mucin and 1.6% hemoglobin were sufficient to kill 50% of the animals. For Haemophilus influenzae type b, 300 bacteria with 5% mucin and 2% hemoglobin were necessary to obtain similar effects.

L2 ANSWER 36 OF 36 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

AB Bacterial meningitis, usually caused by **Neisseria** **meningitidis** **B**, is a disease which still presents considerable problems. All cases of this disease in a hospital in 1979 were reviewed from the bacteriological and clinical point of view. These results were compared with those of a previous study carried out in 1971. Over this period, considerable progress has been made in diagnosis (improved culture techniques) and in the treatment (ampicillin). New techniques, such as bacterial antigen recovery, could reduce bacterial meningitis; new therapeutics could lead to a more favorable prognosis.

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United States Patent *4,673,574*

Anderson **June 16, 1987**

Immunogenic conjugates

Abstract

An immunogenic conjugate which is the reductive amination product of an immunogenic capsular polymer fragment having a reducing end and derived from a bacterial capsular polymer of a bacterial pathogen, and a bacterial toxin or toxoid. The invention also relates to methods for the preparation of the conjugates, a vaccine containing the conjugates which elicits effective levels of anti-capsular polymer antibodies in humans. Also disclosed are methods for inducing active immunization against systemic infection in young mammals caused by bacterial pathogens comprising the administration of an immunogenic amount of the above-described conjugate.

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Field of Search: 424/88,92 260/112 R

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Primary Examiner: Hazel; Blondel

Parent Case Text

This application is a continuation in part of application Ser. No. 298,102, filed Aug. 31, 1981, now abandoned, which is incorporated herein by references.

Claims

I claim:

1. immunogenic conjugate comprising the reductve amination product of an immunogenic capsular polymer fragment having a chain length of from about 10 to about 30 monomeric units and a reducing end, which fragment is derived from the capsular polymer of a Streptococcus pneumoniae or Haemophilus influenzae bacterium, and a bacterial toxin or toxoid.

2. The immunogenic conjugate of claim 1, wherein the capsular polymer is immunogenic in mature humans and less immunogenic in infant humans.

3. The immunogenic conjugate of claim 1, wherein the reductive amination is performed in the persence of cyanoborohydride anions.

4. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is diphtheria toxin or toxoid.

5. The immunogenic conjugate of claim 4, wherein the toxoid is CRM.sub.197.

6. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is tetanus toxin or toxoid.

7. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is a pseudomonas toxin or toxoid.

8. The immunogenic conjugate of claim 1, wherein the toxin or toxiod is a staphylococcus toxin or toxoid.

9. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is a streptococcus toxin or toxoid.

10. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is pertussis toxin or toxoid.

11. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is Escherichia coli toxin or toxoid.

12. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Haemophilus influenzae type b.

13. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 3.

14. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 6.

15. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 12.

16. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 14.

17. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 19.

18. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 23.

19. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is Streptococcus pneumoniae

serotype 51.

20. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Haemophilis influenzae type b.

21. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 6.

22. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 14.

23. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 19.

24. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 23.

25. The immunogenic conjugate of claim 1, wherein the fragment is derived from the capsular polymer by oxidative cleavage.

26. The immunogenic conjugate of claim 1, wherein the fragment is derived from the capsular polymer by periodate.

27. The immunogenic conjugate of claim 1, wherein the fragment is derived from the capsular polymer by hydrolysis of a glycosidic linkage.

28. The immunogenic conjugate of claim 27, wherein the hydrolysis is accomplished enzymatically.

29. The immunogenic conjugate of claim 27, wherein the hydrolysis is accomplished chemically.

30. The immunogenic conjugate of claim 27, wherein the hydrolysis is accomplished by acid.

31. The immunogenic conjugate of claim 12, wherein the fragment elutes on a column of Bio-Gel P-10 at a Ve/Vo ratio of .ltoreq.1.08.

32. The immunogenic conjugate of claim 12, wherein the fragment elutes on a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.09-1.38.

33. The immunogenic conjugate of claim 12, wherein the fragment elutes on a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.39-1.99.

34. An immunogenic conjugate comprising a formalin treated reductive amination product of an immunogenic capsular polymer fragment having a chain length of from about 10 to about 30 monomeric units and a reducing end, which fragment is derived from the capsular polymer of a Streptococcus pneumoniae or Haemophilus influenzae bacterium, and a bacterial toxin or toxoid.

35. The immunogenic conjugate of claim 34, wherein the bacterial toxoid is diptheria toxoid.

36. The immunogenic conjugate of claim 35, wherein the Toxoid is CRM.sub.197.

37. The immunogenic conjugate of claim 34, wherein the bacterial toxin or toxoid is tetanus toxin or toxoid.

38. An immunogenic conjugate of (1) a PRP polysaccharide fragment having reducing terminal groups derived from the capsular polysaccharide of Haemophilus influenzae type b by selective acidic hydrolysis of a portion of the ribosyl ribitol linkages therein and (2) the diphtheria toxin protein CRM.sub.197.

39. The conjugate of claim 38 prepared by the reductive amination of the PRP fragment and protein.

40. The conjugate of claim 38 prepared by reductive amination in the presence of cyanoborohydride anions.

41. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of .ltoreq.1.08.

42. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.09-1.38.

43. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.39-1.99.

44. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of 2.0-2.4.

45. A vaccine that elicits effective levels of anti-capsular polymer antibodies in humans, comprising: the immunogenic conjugate of claim 1.

46. A method for actively immunizing humans against a bacterial pathogen having a capsular polymer, comprising: administering an effective amount of the vaccine of claim 45.

47. A vaccine that elicits effective levels of anti-PRP antibody formations in young warm-blooded mammals comprising an immunogenic amount of the conjugate of claim 41 and a pharmaceutically acceptable carrier.

48. A vaccine that elicits effective levels anti-PRP antibody formations in young warm-blooded mammals comprising an immunogenic amount of the conjugate of claim 42 and a pharmaceutically acceptable carrier.

49. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Streptoccoccus pneumoniae serotype 3.

50. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is Streptococcus pneumoniae serotype 51.

Description

TABLE OF CONTENTS

1. FIELD OF THE INVENTION

This invention relates to the field of novel vaccine compositions, processes for producing them and methods for immunization of young warm-blooded animals, including humans, against infections and disease caused by bacteria, including, for example, Haemophilus influenzae type b, Escherichia coli, Neisseria meningitidis serogroups A and C, Streptococcus pneumoniae serotypes 3; 6, 12, 14, 19, 23 and 51, and Pseudomonas.

2. BACKGROUND OF THE INVENTION

It is known that purified bacterial capsular polymers (CP) generally are immunogenic in mature humans and animals and can be used as vaccines against the corresponding systemic infections. As used in this application, the term "capsular polymers" refers to sugar-containing polymers, such as polymers of sugars, sugar acids, amino sugars, polyhydric alcohols and sugar phosphates, and does not refer to amino acid-containing polymers. These "capsular polymers" are frequently referred to in the medical literature as "capsular polysaccharides", though they may contain linkages other than glycosidic linkages and constituents other than sugars such as those listed above.

The capsular polymers of different bacteria vary widely in immunogenicity in the first year of human life. Some are moderately active, such as Streptococcus pneumoniae serotype 3 and Neisseria meningitidis serogroup A. The susceptibility to systemic infection by encapsulated bacteria is greater in the first year of life. The immunogenic response to many bacterial capsular polymers in children is age dependent, i.e., immunocompetence to CP increases to adult levels by about six years of age.

Among the inactive CP are those of Haemophilus influenzae type b, Streptococcus pneumoniae serotypes 6 and 12, and Neisseria meningitidis serogroup C. Examples of CP's which give an intermediate response in

infants are Streptococcus pneumoniae serotypes 19 and 51.

2.1. INTACT CAPSULAR POLYMERS AS ANTIGENS IN VACCINES

Various investigators have isolated and purified intact capsular polymers which may be useful in or as vaccines. For example, U.S. Pat. No. 4,220,717 describes a process for the isolation and purification of immunologically active polyribosyl ribitol phosphate (PRP) from the capsular polymer of H. influenzae b. Additionally, U.S. Pat. No. 4,210,641 relates to polysaccharide extracts of H. influenzae having an apparent molecular weight greater than 200,000 daltons and composed principally of galactose, glucose and mannose and containing a small amount of osamines.

Several researchers have utilized these and other intact capsular polymers in formulations to achieve better immunological responses. For example, U.S. Pat. No. 4,196,192 discloses a vaccine containing purified intact PRP and whole Bordetella pertussis bacteria. This approach to increasing immunogenicity resulted in enhanced levels of anti-PRP and anti-pertussis antibodies in young mammals.

2.2. VACCINES CONTAINING CONJUGATES

Other researchers have studied conjugation of capsular polymers to carrier proteins in an effort to enhance antibody formation by the so-called "carrier effect". For example, Schneerson et al., Journal of Experimental Medicine 152:361-376 (1980) describes H. influenzae b polymer-protein conjugates disclosed to confer immunity to invasive diseases caused by H. influenzae b. The reference documents the age-related immunological behavior of capsular polymers in infants and seeks to overcome this age-dependence by conjugation of the intact capsular polymer with a variety of proteins, including serum albumins, Limulus polyphemus hemocyanin and diphtheria toxin. The method of conjugation involves the use of a linking agent such as adipic dihydrazide.

Geyer et al., Med. Microbiol. Immunol. 165:171-288 (1979), prepared conjugates of certain Klebsiella pneumoniae capsular polysaccharide fragments to a nitro-phenyl-ethylamine linker by reductive amination, and the derivatized sugar was then attached to proteins using azo coupling.

3. SUMMARY OF INVENTION

The present invention relates to the covalent attachment of capsular polymer fragments derived from bacterial capsular polymers to bacterial toxins or toxoids by means of reductive amination. As used in the present application, the term "toxoid" means a form of a toxin which has the antigenicity of the toxin without its toxicity.

The immunogenic conjugates of the invention are prepared by first forming reducing end groups on fragments of the capsular polymers and reacting these with amine groups of the bacterial toxin or toxoid by reductive amination. The reducing end groups may be formed by any suitable method, including selective hydrolysis, e.g., by acids or enzymes, or by oxidative cleavage, e.g., by periodate. The conjugation is preferably achieved by reductive amination in an aqueous solution containing cyanoborohydride anions.

The immunogenic conjugates of the invention may be formulated with a pharmaceutically acceptable carrier to produce a vaccine which elicits effective levels of anti-capsular antibody formations in young mammals, including humans. The vaccine may be utilized to induce active immunization against systemic infection in young mammals caused by the respective encapsulated bacteria by administering an immunogenic amount of the conjugate to the mammal.

The immunogenic conjugates have been found to be less age dependent than the capsular polymers alone, and are useful for the active immunization of very young warm-blooded mammals against systemic infections by the respective encapuslated bacteria.

Furthermore, the immunogenic conjugates of the invention do not contain potentially toxic linking agents, such as adipic dihydrazide or p-nitro-phenyl-ethylamine, which have been used in conjugating carbohydrate to protein.

Finally, the immunogenic conjugates of the invention contain fragments of capsular polymers, not intact capsular polymers. The highly repetitive structure of capsular polymers may be in part responsible for their failure to expand the capacity for antibody production in infants. A conjugate of intact (highly polymerized) CP and protein may only partially overcome the immunologic disadvantages of CP alone.

On the other hand, the use of capsular polymer fragments on a carrier may circumvent the disadvantages of the repetitive structure. Additionally, the CP determinants of a conjugate having CP fragments are on the average closer to the carrier than are the CP determinants of conjugates having intact CP, and this proximity to carrier may be necessary for a more effective "carrier effect".

A further advantage lies in the use, for the protein carrier, of a bacterial toxin or toxoid against which children are routinely vaccinated, e.g., tetanus or diphtheria. Desired immunity to the toxin or toxoid is induced along with immunity against the pathogens associated with the capsular polymer.

4. DETAILED DESCRIPTION OF THE INVENTION

The conjugates of the invention are formed by reacting reducing end groups of the capsular polymer fragment to primary amino groups of a bacterial toxin or toxoid to yield antigenic determinants of the capsular polymer covalently linked to the carrier protein. The reducing groups may be formed by selective hydrolysis or specific oxidative cleavage.

Antigenic fragments with at least one reducing end can be generated from capsular polymers by a variety of methods, depending upon the structural features of the particular capsular polymer. Limited oxidative cleavage by periodate (or related reagents) will leave aldehydic termini; such an approach will be limited to polymers having vicinal dihydroxy groups on a non-cyclic residue. Hydrolysis of a glycosidic linkage produces a reducing sugar terminus. Such hydrolysis can be most specifically accomplished enzymatically by glycosidases, but this application would be restricted to a relatively few capsular polymers, e.g., Streptococcus pneumoniae 8, for which glycosidases are known. Acidic hydrolysis is commonly used for hydrolysis of glycosidic linkages. The utility of this approach would be limited if the polymer contains acid-sensitive non-glycosidic linkages or if the polymer contains acid-sensitive branch linkages important to the antigenic specificity.

The conjugation is carried out according to the reductive amination process of Schwartz and Gray, Arch. Biochem. Biophys. 181:542-549 (1977). Briefly, the process involves reacting the reducing capsular polymer fragment and bacterial toxin or toxoid in the presence of cyanoborohydride ions, or another reducing agent which will not reduce the reducing ends of interest nor adversely affect the toxin or toxoid capsular polymer. The cyanoborohydrate ions (or their equivalent) act solely as a mild selective reducing agent of the Schiff base intermediate formed between the carbonyl groups of the hydrolyzed capsular polymer fragment and amino groups of the protein. Thus, unlike previously employed conjugation procedures wherein the active molecules are joined by a linking agent which forms a part of the final product, the cyanoborohydride reducing anions utilized herein are not incorporated into the final product. This is important from the standpoint of controlling the potential toxicity of the final product. Evidence of

covalent linkage is demonstrated by the fact that the association between, for example, a PRP moiety and the carrier protein persists despite salting-out of the protein in the presence of 8M urea, which has a great ability to disrupt non-covalent bonds.

Suitable carrier proteins are those which are safe for administration to young mammals and immunologically effective as carriers. Safety would include absence of primary toxicity and minimal risk of allergic complications. Diphtheria and tetanus toxoids fulfil these criteria; that is, suitably prepared, they are non-toxic and the incidence of allergic reactions is well documented. Though the risk of allergic reaction may be relatively significant for adults, it is minimal for infants.

In the "carrier effect" a weak antigen, by being attached to a stronger antigen as carrier (i.e., a heterologous protein), becomes more immunogenic than if it were presented alone. If an animal is previously immunized with the carrier alone, it may become "primed" for an enhanced response not only to the carrier antigen but also the attached weaker antigen. Infants are routinely immunized with tetanus and diphtheria toxoids. Thus, they would be primed for subsequent presentation of a capsular polymer antigen conjugated to either of these toxoids.

In general, any heterologous protein could serve as a carrier antigen. However, certain bacterial toxins such as tetanus and diphtheria may have an additional advantage in that they are composed of two portions, one of which (the "binding" subunit) has a strong affinity for binding to mammalian cell surfaces. Conceivably, conjugation to such a "binding" protein would permit the carried antigen to more effectively initiate responses in cells of the immune system.

The carrier proteins to which the capsular polymer is conjugated may be native toxin or detoxified toxin (toxoid). Also, by relatively recent mutational techniques, one may produce genetically altered proteins which are antigenically similar to the toxin yet non-toxic. These are called "cross reacting materials", or CRMs. CRM.sub.197 is noteworthy since it has a single amino acid change from the native diphtheria toxin and is immunologically indistinguishable from it.

A culture of Corynebacterium diphtheriae strain C7 (197), which produces CRM.sub.197 protein, has been deposited with the American Type Culture Collection, Rockville, Maryland and has been assigned accession number ATCC 53281.

Conjugation of capsular polymer to native toxin may reduce toxicity, but significant toxicity may remain. Thus, further detoxification would be required. Conventional detoxification of protein toxins employs formalin, which reacts with free amino groups of the protein. Residual toxicity may still be a concern. Furthermore, spontaneous retoxification is possible with any particular lot of vaccine and remains an issue of concern with this approach.

Alternatively, native toxin may be detoxified with formalin to produce conventional toxoid before conjugation to capsular polymer. However, the prior formalin treatment reduces the number of free amino groups available for reaction with the reducing groups of the capsular polymer fragment. CRMs, thus, have significant advantages in that they have no inherent toxicity yet none of their amino groups are occupied by the formalin. A further advantage is that no biohazards exist in working with CRMs.

In the case of CRM.sub.197, which is immunologically identical to native toxin, treatment with formalin (though there is no need to detoxify) greatly enhances the immunological response. It is thought that this is due to stabilization of the molecule against degradation by mechanisms of the body and/or aggregation by cross-linking (immunogenicity of particles increases with size).

For all of the above reasons, tetanus and diphtheria toxins are prime candidates for carrier proteins, yet there are others which may also be suitable. Though these others may not have the history of safety found with diphtheria and tetanus, there may be other overwhelming reasons to use them. For instance, they may be even more effective as carriers, or production economics may be significant. Other candidates for carriers include toxins of pseudomonas, staphylococcus, streptococcus, pertussis and Escherichia coli.

Suitable carrier media for formulating a vaccine include sodium phosphate-buffered saline (pH 7.4) or 0.125M aluminum phosphate gel suspended in sodium phosphate-buffered saline at pH 6 and other conventional media.

Generally, vaccines containing from about 5 to about 100 .mu.g, preferably about 10 to 50 .mu.g, are suitable to elicit effective levels of antibody against the capsular polymer in young warm-blooded mammals. Of course, the exact dosage would be determined by routine dose/response experimentation. Several small doses given sequentially would be expected to be superior to the same amount of conjugate given as a single injection.

The vaccines of the invention may be administered by injection to warm-blooded mammals of any age and is especially adapted to induce active immunization against systemic infections in young mammals caused by the pathogens Haemophilus influenzae type b, Escherichia coli, pneumococcus, meningococcus, streptococcus and pseudomonas.

The following are non-limiting examples of methods for the preparation of exemplary immunogenic conjugates of the present invention and their use in vaccines.

5. EXAMPLE: GENERATION OF LARGE, MEDIUM AND SMALL FRAGMENTS OF PRP CONTAINING REDUCING END GROUPS AND CONJUGATION TO CRM.sub.197

The capsular polymer of Hemophilus influenzae type b is a linear polymer with the repeating unit [-3-.beta.-D-ribosyl(1-1)ribitol(5-phosphate)-] (PRP). Generally, hydrolysis of PRP is carried out until the ratio of total to reducing ribose has dropped to 25 or below. The resulting mixture of size fragments may be fractionated by molecular sieve column chromatography to isolate the desired size range of fragments for conjugations. The method for obtaining fragments is as follows:

a. A sample of sodium PRP, (nucleic acid content 0.006%) containing 28.6 milligrams ribose was dissolved with distilled water to make a total volume of 9.2 ml in a 125-ml erlenmeyer flask and chilled in ice.

b. 1.02 ml of 0.1N.sub.2 SO.sub.4 was added.

c. Duplicate samples of 0.01 ml of the acidified PRP were transferred to test tubes held on ice (0-minute)

d. The flask was transferred to a boiling-water bath for 3 minutes, then chilled in an ice-water bath.

e. Step c was repeated (3-minute sample).

f. The samples were assayed for reducing power by the alkaline ferricyanide method standarized with D-ribose.

g. Based on the result (see Table 1), step d was repeated.

h. Step c was repeated (6-minute samples).

i. Step f was repeated.

TABLE 1

Samples	Nanomoles of reducing ribose (av)	Ratio, total ribose/ reducing ribose
0-min	0.42	493
3-min	6.08	34.0
6-min	9.66	21.4

The result (see Table 1) indicated that, assuming the sole mode of hydrolysis had been at the (1-1) glycosidic linkage, the number-average chain length was 21.4 monomeric units, i.e., (ribitol-5-phosphate-3-ribose)

j. 0.102 ml 1N NaOH was added, and the pH was estimated by indicator paper (about pH 6).

k. The neutralized hydrolysate was lyophilized.

l. Bio-Gel P10 (Bio-Rad, Inc.) was equilibrated in 0.1M triethylammonium acetate and poured into a 1.5 cm diameter chromatographic column, giving a gel-bed height of 98 cm.

m. The lyophilized material (step k) was rehydrated with 2.7 ml water, and 0.3 ml of 1M triethylammonium acetate was added. This solution was applied to the column and elution was carried out with collection of 3.5 ml fractions.

n. The elution of ribosyl residues was determined by assay of 0.005-ml samples of each fraction for ribose content by the orcinol reaction with D-ribose as standard.

o. Fractions were combined into 3 pools, L, M, and S as indicated in Table 2, and the pools were assayed for total ribose and reducing ribose:

TABLE 2

Pool	Fractions contained	Total ribose, micromoles	Ratio, total ribose/ reducing ribose	Est. Mn*	Range of Ve/Vo of fraction
L	15-18	577	31.2	11,000	.ltoreq.1.08
M	19-23	744	18.6	6800	1.09-1.38
S	24-34	1180	9.1	3400	1.39-1.99

*on the assumption that the sole hydrolsis was glycosidic.

p. The pools were lyophilized, re-hydrated with 10 ml water, re-lyophilized, re-hydrated with 1.5 ml water. 1.2 ml of the last solutions were transferred to microcentrifuge tubes and lyophilized in preparation for the conjugation reactions.

Conjugation of CRM.sub.197 to Reducing Fragments of PRP a. To the microcentrifuge tubes containing lyophilized fragments, L, M, and S and an empty tube (C or control) were added potassium phosphate buffer pH 8, 2.7 milligrams CRM.sub.197, and 4 milligrams sodium cyanoborohydride, such that the final volume was 0.2 ml and the phosphate buffer was at 0.2M.

b. The tubes were incubated at 37.degree. C. with daily mixing.

c. After 18 days the tubes were centrifuged 2 minutes at 7000 G.

d. After determination that the majority of protein was in the precipitates, the precipitates were washed four times with .ltoreq.1 ml water.

e. The washed precipitates were made 8M in urea and warmed to 50.degree. C., dialyzed against saline overnight at 4.degree. C., and centrifuged. The supernates were separated and made 95% saturated in ammonium sulfate, held overnight at 4.degree., and centrifuged. The resulting precipitates were washed 3 times with 0.4 ml of 95% saturated ammonium sulfate, and suspended with 1 ml water. These colloidal suspensions were labeled CRM.sub.197 -PRP-L, -M, -S, and CRM.sub.197 -C, respectively.

f. The preparations were assayed for protein by means of the Folin phenol reaction with bovine albumin as standard and for ribosyl residues with the orcinol reaction and D-ribose as standard. The results are given in Table 4. The preparations were assayed for PRP antigenic activity by their ability (at concentrations of 50 micrograms protein/ml) to inhibit the binding of labeled native PRP to human anti-PRP antibody (Table 3).

TABLE 3

Preparation tested	% Antigen bound	antigenic activity, ng PRP equivalence/.mu.g protein
none	28.1	--
> native PRP, 0.5 ng/ml	6.7	--
> native PRP, 5 ng/ml	0.94	--
CRM.sub.197 - C	34.3	0.0
CRM.sub.197 - PRP-S	2.0	0.1
CRM.sub.197 - PRP-M	2.5	0.08
CRM.sub.197 - PRP-L	3.9	0.006

Thus, all the tested conjugates of CRM.sub.197 with PRP fragments were antigenically active, while the control preparation in which CRM.sub.197 was exposed to cyanoborohydride in the absence of PRP fragments was inactive as expected.

The preparations were assayed for immunogenicity in rabbits in comparison with high molecular weight purified PRP, and the results are given in Table 4. Rabbits given the PRP control or the CRM.sub.197 -C control made barely detectable increases in anti-PRP antibody. Rabbits given any of the three CRM.sub.197 -PRP conjugates made progressive increases after each injection; the titers after the third injection were 1000-fold greater than prior to immunization. In an experiment not illustrated a simple mixture of CRM.sub.197 and PRP fragment preparation L was assayed in rabbits and found not to elicit anti-PRP antibody.

TABLE 4

ANTI-PRP ANTIBODY RESPONSE TO CONJUGATED AND CONTROL VACCINES OF WEANLING RABBITS PRIMED WITH ORDINARY DIPHTHERIA TOXOID*

Rabbit	Vaccine**	Pentose/protein ratio	Anti-PRP Antibody, ng/ml, at age in weeks 7***	8***	9***	10
1	PRP(MW 10.sup.5)		<10	12	28	40
2	"		<10	<10	27	26
3	CRM.sub.197 -C (control)	--	35	25	31	36
4	"		16	34	40	48
5	CRM.sub.197 -PRP-S	0.015	19	980	26,000	49,000
6	"		<10	84	23,000	31,000
7	CRM.sub.197 -PRP-M	0.0069	<10	37	2,500	11,000
8	"		23	11,000	49,000	150,000
9	CRM.sub.197 -PRP-L	0.0020	14	73	3,700	26,000
10	"		<10	340	9,800	76,000

*The rabbits were New Zealand Whites obtained from Dutchland Farms immediately after weaning. At six weeks of age each was injected subcutaneously (s.c.) with 40 Lf of diphtheria toxoid (Massachusetts Dept of Public Health) contained in 0.5 ml of 0.0 125 M aluminum phosphate pH (alum).

**The PRP vaccine was 30 .mu.g PRP lot 17 contained in 0.1 ml saline. The other vaccines were 25 .mu.g protein contained in 0.5 ml alumn.

***Injections of the indicated vaccine were given (s.c.) immediately afte bleeding. There were two rabbits per vaccine. Listed are individual titers, determined by radioantigen binding with .sup.3 Hlabeled native PRP.

The protective potential of the anti-PRP antibodies induced by the conjugates was evaluated by testing the bactericidal activity of the rabbit sera of Table 4. The bactericidal titers were determined against H. influenzae b strain Eag by the methods of Anderson et al, Journal of Clinical Investigation, Volume 65, pages 885-891 (1980). Table 5 shows that before vaccination the sera were unable to kill the bacteria (reciprocal titers <2). After three injections the reciprocal titers of the rabbits receiving the CRM.sub.197 - PRP conjugates had risen to 16 or greater while titers of the rabbits receiving the CRM 197 control

remained at <2.

TABLE 5

Bacterial Titers Against H. influenzae b Strain Eag of Sera of Weanling Rabbits Vaccinated With CRM.sub.197 of Its Conjugates With Oligosaccharides S, M, and L of PRP*

Reciprocal serum dilution for > 90% Killing

Rabbit	Vaccine given	Pre-vaccination	After 3 injections
3	CRM.sub.197 control	<2	<2
4	CRM.sub.197 control	<2	<2
5	CRM.sub.197 -PRP-S	<2	128
6	CRM.sub.197 -PRP-S	<2	.gtoreq.256
7	CRM.sub.197 -PRP-M	<2	16
8	CRM.sub.197 -PRP-M	<2	64
9	CRM.sub.197 -PRP-L	<2	64
10	CRM.sub.197 -PRP-L	<2	32

*Same vaccinations as described in Table 4.

6. EXAMPLE: VARIATION OF PRP FRAGMENT RATIO TO CRM.sub.197

In this example, the ratio of PRP fragment S to CRM.sub.197 was varied and the conservation of antigenic activity of the CRM.sub.197 component was examined in addition to the PRP component.

Preparation of CRM.sub.197 -PRP-S#2, A and B.

a. To microcentrifuge tubes A and B were added 0.15 ml each of the solution of fragments S described above, i.e., steps o and p. The solutions were lyophilized.

b. Tube A received 0.015 ml 2M potassium phosphate buffer pH 8, 0.1 ml of CBM.sub.197 5 mg/ml in 0.01M sodium phosphate buffer pH 7, and 0.015 ml of sodium cyanoborohydride 200 mg/ml.

c. Tube B received 0.002 ml of the pH 8 buffer and 0.1 ml of the CRM.sub.197 solution. The resulting solution was lyophilized. The solids were suspended with 0.015 ml water, and 0.002 ml of the pH 8 buffer were added.

d. Tubes A and B were incubated at 37.degree. C. for 13 days. To tube B an additional 0.002 ml of cyanoborohydride was added. Both tubes were incubated at 37.degree. C. for an additional 3 days. (Note that due to the reduced reaction volume, the concentrations of reactants in B were higher than A.)

e. To A was added 0.06 ml water and 0.8 ml saturated ammonium sulfate (SAS). To B was added 0.175 ml

water and 0.8 ml SAS.

f. The tubes were incubated 1 hour at 0.degree. C. and centrifuged 20 minutes at 8000 G. The supernates were removed.

g. The precipitates were washed by suspension in 1 ml of 80% SAS, centrifugation at 8000 G 20 minutes, and removal of the supernates.

h. The precipitates were suspended with 0.1 ml water, and 0.4 ml SAS was added.

i. Same as step f.

j. Same as step g.

k. The precipitate in B was dissolved with 0.084 ml 9.5M urea (estimated final concentration 8M); 0.1 ml water and 0.8 ml SAS were added, and the precipitate was isolated as in step f. This precipitate was washed as in step g.

1. The precipitates in A and B were suspended with 0.2 ml water. The suspensions were separated into soluble (s) and insoluble (i) fractions by centrifugation 30 minutes at 8000 G, and the s fractions (supernates) were made 0.01M sodium phosphate buffer pH and reserved.

m. The i fractions (precipitates) were rendered more soluble as follows: they were made 8M in urea, which was then gradually removed by dialysis against 0.01M sodium phosphate buffer pH 7. The resulting solutions were recombined with the respective s fractions.

n. Preparations A and B were tested for protein content with the Folin phenol reagent and for PRP antigenic activity by the assay described above. Both had PRP activity; B exceeded A by about 13-fold, as shown below:

Preparation	ng PRP equivalence/.mu.g protein
CRM.sub.197 -PRP-S#2,A	0.038
CRM.sub.197 -PRP-S#2,B	0.50

o. Preparations A and B were tested for CRM antigenicity (activity as diphtheria toxoid (DT)) by inhibition of the binding of antibody to a sample of purified DT furnished by the Massachusetts Department of Public Health. Both had activity roughly equal to the DT on a weight basis; B exceeded A by about 4-fold, as shown below.

Inhibitor tested	Antibody bound, A.sub.400 per .mu.g protein	.mu.g DT equivalence
None	2.43	
DT, 0.5 .mu.g/ml	2.56	
DT, 5 .mu.g/ml	1.93	

DT, 50 .mu.g/ml			0.96			
CRM.sub.197 -PRP-S#2,A,50 .mu.g/ml			1.25		0.52	
CRM.sub.197 -PRP-S#2,B 5 .mu.g/ml			1.67		2.0	

p. Preparations A and B were suspended in alum at 16 ug protein 1 ml, and three 0.5 ml injections were given to rabbits in the protocol described in Table 4 (except the animals were 8 weeks old at the onset and not primed by previous injections of diphtheria toxoid). The sera were tested for antibodies in the binding assay described in step o. Both A and B elicited antibodies to DT as well as to PRP, as shown in Table 6. Separate control experiments showed that similar rabbits housed in the same quarters did not display such increases in anti-DT antibody values in the absence of being injected with CRM.sub.197 preparations.

TABLE 6

Rabbit	Injected antibody to	Assay for	Antibody values at age 8 wk	9 wk	10 wk	11 wk
5	A	PRP, ng/ml	47	60	210	13,500
		DT, A.sub.400	0.136	0.168	1.28	3.81
6	A	PRP	21	25	19	420
		DT	0.072	0.049	0.262	3.23
7	A	PRP	<20	20	2000	10,500
		DT	0.155	0.134	0.155	0.676
3	B	PRP	<20	27	1600	4900
		DT	0.075	0.061	0.227	2.45
8	B	PRP	23	<20	2900	26,000
		DT	0.065	0.023	0.231	2.07

7. EXAMPLE: CONJUGATION OF VERY SMALL FRAGMENTS OF PRP TO DIPHTHERIA TOXIN, DIPHTHERIA TOXOID AND CRM.sub.197

Generation of Very Small Fragments of PRP Containing Reducing End Groups

a. A 12 ml solution of PRP lot 20 was made 0.1M in HCl at 0.degree. C. and sealed in a glass flask (0 minute).

b. The flask was transferred to a boiling-water bath for 4 minutes, then chilled in an ice water bath.

c. A small amount of resulting white colloid was removed by extraction with ether and the resulting clear solution was lyophilized.

d. Bio-Gel P10 (Bio Rad, Inc.) was equilibrated in 0.01M ammonium acetate and poured into a 1.5 cm diameter chromatographic column, giving a gel bed height of 98 cm.

e. The lyophilized material was rehydrated with 1.5 ml water and neutralyzed with NH.sub.4 OH. This

solution was applied to the column and the elution was carried out.

f. Fragments eluting at Ve/Vo range of 2.0-2.4 were collected and designated fraction vs.

g. Steps a-f were repeated to double the supply of fraction vs.

h. The combined vs fractions were lyophilized, rehydrated to yield 4 ml of a solution containing a total of 47 umoles of reducing sugar activity when assayed by the alkaline ferricyanide method standardized with D-ribose.

Preparation of Conjugates of PRP-vs Fragments to Native Diphtheria Toxin, Native Diphtheria Toxoid and CRM.sub.197

The following proteins are used as carriers in the present example:

(1) DTx--purified diphtheria toxin, lot 1, obtained from the Massachusettes Public Health Biologic Laboratories. Partial detoxification is accomplished by the linking to PRPvs. Residual toxicity is removed by formalin treatment in the presence of lysine by the method of Pappenheimer et al., Immunochemistry, 9:891 (1972).

(2) DTd--conventional (formal) toxoid, lot DCP-27, also obtained from the Massachusettes laboratories.

(3) CRM.sub.197 --antigenically mutated version of the toxin protein, antigenically indistinguishable from toxin but non-toxic.

The conjugation method is as follows:

a. Protein, potassium phosphate buffer (pH 8.0 at 25.degree. C.) and PRPvs were combined in glass centrifuge tubes in the manner set out below.

Solution	Protein	Buffer	PRPvs
(1)	30 mg DTx	0.24 .mu.mol	20 .mu.mol
(2)	30 mg DTd	0.24 .mu.mol	20 .mu.mol
(3)	10 mg CRM.sub.197	0.08 .mu.mol	6.7 .mu.mol

b. The solutions were lyophilized, and the lyophiles were dissolved with NaCNBH.sub.3 solution, 2% w/v in water as tabulated below.

Solution	2% NaCNBH.sub.3
(1)	1.2 ml
(2)	1.2 ml
(3)	0.4 ml

c. The tubes were incubated at 37.degree. C.

d. After 14 days, four volume-equivalents of saturated ammnonium sulfate were added. These suspensions were held 3 hours at 0.degree. C., then centrifuged 20 minutes at 9000 G.

e. The precipitates were washed twice each with 10 ml of neutral 70% saturated ammonium sulfate.

f. The washed precipitates were dissolved with a minimal volume of 9.5M urea and dialyzed against 0.067M sodium phosphate buffer, pH 7.8.

Formalin Treatment of the Conjugates

a. The conjugates were further dialyzed against sodium phosphate buffer which also contained 0.025M lysine. (Small samples were reserved for toxicity testing prior to formalinization).

b. Formalin was added to a final concentration of 0.2% v/v.

c. After 17 days incubation at about 24.degree. C. the solutions were extensively dialyzed against the sodium phosphate buffer.

d. Centrifugation was performed to remove small amounts of insoluble material.

Processing to Achieve Final Container Products

a. Antigen solutions (1)-(3) in isotonic sodium phosphate buffer were passed through 0.22-micron "Millex" filter units (Millipore Corp.) and injected into bottles containing sterile phosphate buffered saline.

b. The preparations were assayed for protein using the Lowry method.

c. Thimerosal was filtered and injected into the solution as 1/100 volume of a freshly made 1% w/v solution. Samples of 10 ml were taken for a sterility test. The bottles were attached to a manually operated sterile single use filling device (Multiple Additive Set, Travenol Laboratories). 2-ml glass vials were filled, stoppered, sealed, and immediately transferred to storage at 4.degree. C.

Assays on Conjugate Preparations

a. Phosphate content of the protein fraction

PRP is composed of the repeating unit ribosyl-ribitol-phosphate. Thus colorimetric assay of phosphate in the fraction precipitable by 5% tricholoracetic acid (TCA) is a sensitive index of the incorporation of PRP fragments into the protein.

Samples containing 100 .mu.g protein were made 5% in TCA in a volume of 3 ml, held 20 minutes on ice, and centrifuged 15 minutes at 4.degree. C. at G. The precipitates were washed with an additional 3 ml of 5% TCA, then with 5 ml ethanol. The washed precipitates were ashed to convert organic phosphate to inorganic phosphate (Pi), and the Pi was quantified by the method of Chen et al., Anal. Chem., 28:1756 (1956). The results were as follows:

Sample	nmol Pi/ .mu.g protein	Implied average no. of PRP repeating units/protein
(1) DTx-PRPvs	0.11	6.8
(2) DTd-PRPvs	0.10	6.2
(3) CRM.sub.197 -PRPvs	0.10	6.2

b. Electrophoretic Analysis

Samples of the conjugated antigens were analyzed by mercaptoethanol-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (ME-SDS-PAGE) in the same gel alongside the respective starting carrier protein preparations.

DTd-PRPvs, like the DTd, displayed a disperse band at MW 61,000 daltons. In contrast, DTx-PRPvs and CRM.sub.197 -PRPvs differed greatly from the starting proteins. The protein of these two conjugates collected either at the beginning of or in the stacking gel (4% acrylamide) or at the beginning of the separating get (10% acrylamide). Thus, the conjugates appear to have been converted into macromolecular aggregates, presumably by cross-linking from the formalin treatment. DTd-PRPvs also contains some aggregated material.

c. PRP Antigen Equivalence per Unit Protein

The capacity of the conjugates to bind anti-PRP antibody was determined by the inhibition of the binding of labeled PRP by human anti-PRP antiserum, calibrated with PRP lot 19. (Because protein-bound polymer fragments cannot be assumed to bind to antibody in a weight-equivalent fashion to the high molecular weight polymer, quantitative chemical composition cannot be inferred from these data.)

Sample	% Inhibition of .sup.3 H-PRP bound	ng PRP equivalence/ .mu.g protein
PBS control	(0)	--
PRP 19, 0.5 ng/ml	6.7	--
PRP 19, 5 ng/ml	32	--
PRP 19, 50 ng/ml	90	--
DTx-PRPvs, 5 .mu.g protein/ml	24	0.5
DTd-PRPvs, 5 .mu.g protein/ml	48	2.2
CRM.sub.197 -PRPvs, 5 .mu.g protein/ml	38	1.4

d. Diphtheria Toxoid Antigenic Equivalene Per Unit Protein

Retention of the capacity of the preparations to react with anti-DTd antibody was determined by inhibition of an enzyme-linked immunosorbent assay (ELISA) in which purified DTd is attached to the assay tube (solid phase). Inhibition of antibody binding to the attached DTd is calibrated by the same DTd used in the fluid phase.

Sample	% Inhibition of Antibody Binding	.mu.g DTd equivalence/ ug protein
PBS control	(0)	--
DTd, 5 .mu.g protein/ml	24	--
DTd, 50 .mu.g protein/ml	50	--
DTx-PRPvs, 50 .mu.g protein/ml	46	0.68
DTd-PRPvs, 50 .mu.g protein/ml	58	2.1
CRM.sub.197 -PRPvs, 50 .mu.g protein/ml	26	0.11

e. Diphtheria Toxic Activity

Samples of the original DTx and the conjugate DTx-PRPvs before and after formalin treatment were titrated for toxic activity by injection into the skin of a non-immune adult rabbit. DTx at doses of 0.002 .mu.g and 0.02 .mu.g produced the expected dermal lesions. DTx-PRPvs prior to formalin treatment produced dose-dependent lesions such that 0.2 .mu.g was approximately equal to 0.002 .mu.g DTx (100-fold reduction in toxicity by the conjugation). After formalin treatment, lesions were not generated by doses as high as 2 .mu.g (at least 1000-fold reduction relative to DTx). Doses up to 2 .mu.g of conjugates DTd-PRPvs and CRM.sub.197 -PRPvs were tested similarly and generated no lesions.

f. Induction of Anti-PRP Antibody Responses in Weanling Rabbits, Measured by Radioantigen binding

The antigens were mixed with an aluminum phosphate adjuvant (0.0125M Al, pH 6) such that a 0.5 ml dose contained 25 .mu.g protein. Two rabbits (for each antigen) were given three weekly injections beginning at age 7 weeks; the rabbits had been injected with DTd alone at age 5 weeks, for a hypothetical "carrier priming" effect. All the animals (rabbits 1-6) had anti-PRP rises in an anamnestic pattern, with titers of at least 10 .mu.g/ml after the third vaccination. Antigens CRM.sub.197 -PRPvs and DTd-PRPvs were also tested in pairs of rabbits that had not been "primed" with DTd. These (rabbits 7-10) produced strong anti-PRP reponses similar to those in the "primed" rabbits.

g. Induction of Anti-DTd Antibody Response in Weanling Rabbits, Measured by ELISA

The anti-DTd antibody responses in the same "unprimed" rabbits (7-10) of the preceding subsection are as follows: Rises were roughly 10-fold after the second injection and another 2-to 5-fold after the third.

h. Sterility of the Sample Preparations

The samples were found to be sterile as determined using Fluid Thioglycollate (BBL cat. no. 11260, lot

D4D LKL) as the growth medium.

8. EXAMPLE: USE OF PRP FRAGMENTS CONJUGATED TO DIPHTHERIA TOXOID AND CRM.sub.197 AS VACCINES IN YOUNG HUMANS

Two groups of 8 children in the age range of 1 to 2 years old, (and specifically exempting children receiving routine vaccination with diphtheria toxoid protein at age 18 months) were given primary and secondary vaccinations as follows: Group I received injections of CRM.sub.197 -PRPvs, preparation as described in the preceding section, (25 .mu.g protein in saline, subcutaneously); Group II received injections of DTd - PRPvs, preparation as described in the preceding section, (25 .mu.g protein in saline, subcutaneously).

In the first visit, pre-vaccination blood specimens were taken; the child was vaccinated, then observed for 20 minutes for any sign of an anaphylactic reaction. In the second visit the procedure of the first visit was repeated. In the third visit, a post-secondary blood specimen was taken. Two of the children, one from each group, after consultation with the parents, were given a third vaccination to try to raise the antibody against PRP to protective levels. The interval between vaccinations was 1.+-.1/2 month.

Group III consisted of children about 18 months old receiving a vaccine simultaneously with diphtheria toxoid protein in a separate site. This group contained 2 children; one received the CRM.sub.197 -PRPvs vaccine, the other received the DTd-PRPvs vaccine.

Symptoms were recorded for four successive days, with measurements of temperature, notation of behavioral indications of systemic illness and observations of inflammation at the injection site. These symptoms are summarized in Table 7.

TABLE 7

ADVERSE REACTIONS TO PRP-VS CONJUGATES TO CRM.sub.197 AND FORMAL DIPHTHERIA TOXOID

		Injection		
Vaccine	Symptom	Pri-mary	Secon-dary	Terti-ary
CRM.sub.197 -PRPvs				
	Fever	1/8	0/8	0/1
	Unusual behavior	0/8	0/8	0/1
	Local inflammation	1/9*	2/9	0/1
	Local pain	1/9*	1/9	0/1
DTd-PRPvs	Fever	0/8	0/8	0/1
	Unusual behavior	0/8	0/8	0/1
	Local inflammation	1/9*	0/9	0/1
	Local pain	1/9	1/9	0/1

*Includes one child who received diphtheria toxoid protein simultaneously in a separate site. No local symptoms were found. Systemic symptoms are not noted since these could not be distinguished from an effect of the diphtheria toxoid protein vaccine.

After CRM.sub.197 -PRPvs vaccination, one child had mild fever (99.8.degree. C.) on the evening of

primary vaccination; there was an instance of mild local inflammation once each after a primary, a secondary, and the one tertiary vaccination. After DTd-PRPvs there was an instance of local inflammation after one primary and one secondary vaccination. The administration of the vaccines was otherwise apparently free of adverse reactions.

Serum Antibody Reponses

Antibodies to PRP as well as IgG antibodies to diphtheria toxoid were determined. After vaccination with CRM.sub.197 PRPvs a consistent anti-PRP response pattern was seen. See Table 8. There was a distinct rise after the primary injection, usually an even larger rise after the secondary injection, and a large rise after the one tertiary. The final titers greatly exceeded those that have been produced by vaccination with PRP alone and greatly exceeded the accepted estimated protective minimal level of 0.15 .mu.g/ml. The enhanced response was particularly evident in the four children under 18 months of age, where the response to PRP alone is generally inadequate for protection, and the geometric mean of the final titers in these four (8.4 .mu.g/ml) is 175 times that found after vaccination of children 12-17 months old with PRP vaccine alone. The child receiving the primary vaccination simultaneously with diphtheria toxoid protein vaccine also had an excellent response.

IgG antibodies to diphtheria toxoid increased in 6 of 8 children (as well as in the 9th, who also received diphtheria toxoid as part of the treatment). The antibody levels often increased so greatly that the dilution of post-vaccination serum used (1/1000) was insufficient to show the full extent of the rise.

After vaccination with DTd-PRPvs anti-PRP responses generally increased after both primary and secondary vaccination. (See Table 9). However, there were two children (12 and 14 month old) in whom no response was detected; and one child did not approach the protective level until given a third injection. The child receiving the primary vaccination simultaneously with diphtheria toxoid protein had an excellent response. Rises in IgG antibody to the diphtheria component were found in all children.

TABLE 8

ANTIBODY RESPONSE TO CRM.sub.197 -PRPvs

Subject	Age at primary vaccination	Serum sample	Serum antibody, .mu.g/ml anti-PRP IgG	anti-DTd
1	12 mo	pre-vac	2.0	1.1
		post-1	4.5	>10
		post-2	18	>10
2	13 mo	pre-vac	<0.006	0.38
		post-1	0.040	1.7
		post-2	0.35	2.2
		post-3	4.8	1.9
3	14 mo	pre-vac	<0.020	4.5
		post-1	0.12	3.3
		post-2	2.0	4.3
4	16 mo	pre-vac	0.025	0.06
		post-1	0.92	5.7
		post-2	29	9.1
5	27 mo	pre-vac	0.025	3.0
		post-1	10	>10
		post-2	58	>10
6	29 mo	pre-vac	0.13	6.1
		post-1	22	6.9
		post-2	180	7.4
7	30 mo	pre-vac	2.2	6.5
		post-1	28	>10

		post-2	50	>10
8	30 mo	pre-vac	1.3	4.8
		post-1	6.5	>10
		post-2	78	>10
9	18 mo*	pre-vac	0.34	3.1
		post-1	1.4	>10
		post-2	8.2	>10

*First injection of CRM.sub.197 -PRPvs given simultaneously with diphtheria toxoid protein vaccine in a separate site

TABLE 9

ANTIBODY RESPONSE TO DTd-PRPvs

Subject	Age at primary vaccination	Serum sample	Serum antibody, .mu.g/ml anti-PRP IgG	anti-DTd
1	12 mo	pre-vac	<0.020	0.060
		post-1	<0.020	10
		post-2	<0.020	10
2	12 mo	pre-vac	0.055	0.03
		post-1	0.080	3.1
		post-2	1.8	10
3	13 mo	pre-vac	<0.006	1.1
		post-1	<0.006	10
		post-2	0.023	10
		post-3	0.120	10
4	14 mo	pre-vac	<0.020	3.0
		post-1	<0.020	5.1
		post-2	<0.020	3.8
5	19 mo	pre-vac	0.060	8.0
		post-1	0.12	10
		post-2	0.76	10
6	26 mo	pre-vac	<0.020	6.9
		post-1	0.060	10
		post-2	0.94	10
7	27 mo	pre-vac	1.4	6.1
		post-1	7.4	10
		post-2	21	10
8	28 mo	pre-vac	<0.020	8.7
		post-1	0.63	10
		post-2	8.0	10
9	18 mo*	pre-vac	1.9	0.11
		post-1	2.9	10
		post-2	11	10

*First injection of DTdPRPvs given simultaneously with diphtheria toxoid protein vaccine in a separate site

This example shows that injections of conjugates of the H. influenzae b capsular polymer fragment to diphtheria toxoid and CRM.sub.197 is apparently harmless. CRM.sub.197 -PRPvs vaccination gave a clear indication of an enhancement of the anti-PRP response by the carrier effect --appreciated not only by the high titers but by the rises after secondary vaccination.

DTd-PRPvs had a less impressive enhancement. A likely explanation is that while CRM.sub.197 -PRPvs is a multimolecular aggregate, DTd-PRPvs is present mainly in unimolecular form similar to the original toxoid.

9. EXAMPLE: CONJUGATION OF CAPSULAR POLYMER FRAGMENTS OF STREPTOCOCCUS

PNEUMONIAE TO CRM.sub.197

Several other bacteria resemble H. influenzae b in that they cause sepsis and meningitis, particularly in infants; they have polymer capsules, antibodies to which are protective; and their capsular polymers are immunogenic in mature humans but not in infants. An important example is Streptococcus pneumoniae (Sp) serotype 6. It causes not only the life-threatening infections mentioned above but also is a highly prevalent cause of otitis media in children. (Gray et al, Journal of Infectious Diseases, Volume 142, pages 923-33, 1980).

The approach described for PRP is also applicable to any capsular polymer in which reducing groups can be generated by selective hydrolysis with retention of antigenic specificity. In the following non-limiting example, capsular polymer fragments were made from the Sp. 6 capsular polymer by selective acid hydrolysis and were conjugated to CRM.sub.197. The product retained antigenic specificity for both the Sp capsular polymer and the CRM.sub.197 component.

Generation of Reducing Fragments From Capsular Polymers (CP)

1. A sample of the CP of Sp. 6 (Danish type 6A, Eli Lilly Co.) was assayed for total hexose by the phenol-sulfuric acid method standardized with D-glucose and for reducing activity by the alkaline ferricyamide method also standardized with D-glucose.

2. A Pyrex tube received 3.3 mg Sp. 6 CP dissolved with 0.66 ml water. The sample was chilled to 0.degree. C., 0.073 ml of 0.1N HCl were added, and the tube was sealed.

3. The tube was immersed 10 minutes in a boiling water bath, then rechilled to 0.degree. C. A small sample was assayed for reducing activity as described in step 1:

CP	Time heated at 100.degree. C.	Total hexose/ reducing hexose
Sp. 6	0 minutes	>350
	10 minutes	6.5

4. The hydrolyzed preparation (minus the 2% used for assay) was lyophilized. The dried material was dissolved with 0.1 ml water, transferred to microcentrifuge tube, and lyophilized again.

Conjugation to CRM.sub.197 1. To the re-dried hydrolysate was added 0.004 ml of 2 M potassium phosphate buffer pH 8 and 1 mg of CRM.sub.197 dissolved in 0.2 ml of 0.01M sodium phosphate buffer pH 7. The resulting mixture was lyophilized and resuspended with 0.05 ml water (estimated total volume 0.063 ml).

2. To the tube was added 0.007 ml of sodium cyanoborohydride at 200 mg/ml, and the preparation was incubated 18 days at 37.degree. C.

3. 0.6 ml 80% saturated ammonium sulfate (SAS) was added.

4. The tube was incubated 1 hour at 0.degree. C. and centrifuged 15 minutes at 8000 G; the supernate was

removed.

5. The precipitate was washed by suspension in 0.6 ml of 80% SAS buffered at pH 8 with 0.01M sodium phosphate, followed by centrifugation 15 minutes at 8000 G.

6. The precipitate was suspended with 0.02 ml of 0.5M Na.sub.2 HPO.sub.4 and 0.2 ml 9.5M urea.

7. 1 ml SAS was added, the precipitate was isolated as in step 4 and suspended in urea at about 8M as in step 6.

8. The suspension was centrifuged 15 minutes at 8000 G.

9. The supernate was separated and dialyzed against 0.01M sodium phosphate buffer pH 7 at 4.degree. C.

10. The dialyzed preparations, designated CRM.sub.197 -Sp. 6 was assayed for the following:

protein by the Folin phenol reaction;

Sp antigenicity by inhibition of binding of antibody to radiolabeled Sp CP (as described for PRP in Table 3);

CRM.sub.197 antigenicity by the inhibition of antibody binding to diphtheria toxoid (DT) (as described in step o of the description of CRM.sub.197 -PRP-S#2); and

anti-CP immunogenicity by inhibition of the binding of antibody to diphteria toxiod (DT) (as described in step p of the description of CRM.sub.197 -PRP-S#)

Preparation	ng CP equivalance/ .mu.g Protein	.mu.g DT equivilance/ .mu.g protein
CRM.sub.197 Sp. 6	0.4	0.36

TABLE 10

ANTI-CP IMMUNOGENIC RESPONSE OF WEANLING RABBITS WITH CONTROLS AND CONJUGATES OF STREPTOCOCCUS PNEUMONIAE SEROTYPE 6 FRAGMENTS OF CRM.sub.197

	Vaccinated Rabbit With*	Percent .sup.14 C-CP Bound in Samples at age** 6 wk	8 wk	10 wk	11 wk
1	Sp 6 CP, 25 .mu.g	6	6	7	7
2	"	6	13	13	11
3	Sp 6 bacteria 25 .mu.g	4	10	12	16
4	"	8	12	22	21
5	CRM.sub.197 Sp 6, 25 .mu.g	4	6	30	49
6	"	4	8	30	54

*Injected subcutaneously just prior to taking serum samples. Serum sample were taken at age 6, 8 and 10 weeks.
**25 ul serum incubated with 2 nCi .sup.14 Clabelled CP.

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